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**An Investigation of the Cytotoxicity and Mechanisms of Cell Death induced by
Novel Antitumor Platinum and Palladium (IV) Complexes**

A Senior Honors Thesis in the Department of Biology, Sweet Briar College

by

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Abstract: Cisplatin is a platinum-based drug successfully used to treat a variety of cancers. Its success has stimulated much research into other platinum compounds with antitumor activity. This paper describes research comparing and investigating the cytotoxic properties of several novel homoleptic diimine Pt and Pd based compounds produced by Dr. Robert Granger. Cytotoxicity on matched cell lines of normal and transformed cells was assessed and compared by assay with MTT. The compounds showed selective toxicity to transformed cells over normal cells. Investigations of the biochemistry of cell death using the TUNEL method and gel electrophoresis suggests that the novel Pt and Pd compounds induce apoptosis in cells in culture. These results suggest that the novel compounds might have promise as anticancer chemotherapeutic agents and certainly warrant further research.

Introduction:

Most Americans have had some contact with cancer at some point in their lives. Cancer is currently the second leading cause of death of Americans; one in four will die from it and nearly one in two will be affected by it (Cooper 1992). The field of cancer research is obviously quite active, and the need for improved treatments for cancer always present. Cisplatin is a platinum based drug that has been used with much success in the treatment of cancer. However, it has its drawbacks, including toxicity to the patient and resistance by some tumors. Since its discovery over three decades ago research has been directed into developing other similarly metal based compounds for use in chemotherapy. This paper discusses an investigation into the cytotoxic properties of several new platinum and palladium based compounds first synthesized in the mid 1990s by Dr. Robert Granger. Although these compounds differ significantly from cisplatin in structure, they are based on platinum and palladium, and have proven biological activity.

Causes of Cancer

Mutation is a natural part of the process of DNA replication. In order for organisms to grow and maintain themselves, the DNA in their cells must be replicated at a very rapid rate. Although mechanisms do exist to proofread the replication process, sometimes mistakes are made. Not all mutations are harmful - most are silent or simply contribute to a species' genetic variability. Most mutations are repaired quickly within a cell. Other mutations persist and are passed on to daughter cells, when the cell divides.

Some mutations, in proto-oncogenes (which become oncogenes) or in tumor suppressor genes, can be detrimental to the health of the organism. A proto-oncogene is a gene that promotes cell division, and a mutation in one can over stimulate proliferation of a cell. In contrast, a tumor suppressor gene normally acts to stop cell division. Some tumor suppressor genes are "checkpoint" genes that produce proteins that insure that cells distribute their genetic material evenly when they divide. These proteins can act to stop an abnormally dividing cell until the problem can be repaired and the cell can continue normal cell division. Other tumor repressor genes regulate the cell cycle by inducing apoptosis in a cell that has a mutation that is causing it to divide abnormally, such as an aneuploidy. These types of tumor suppressors, including p53 and bcl-2, are of particular interest to cancer researchers because mutations in them can increase cancer rates and also prevent those cancers from being susceptible to anti-tumor drugs that work by damaging DNA.

The true mark of cancer is invasiveness. Malignancy is defined as the ability of a tumor to invade surrounding tissues (Nowell 1976). It takes about a half dozen mutations for a cell to become malignant or cancerous (Cooper 1992). The initial mutations usually occur in genes that increase cell growth or prevent cell death (Cooper 1992). These mutations allow the cell to escape the normal factors that regulate cell growth and division; at this stage the cell is termed "neoplastic" (Nowell 1976). These new mutant cells may be localized and benign (Cooper 1992). However, when a cell divides more rapidly than its neighboring (normal) cells, it is likely to develop more mutations (Cooper 1992). Subsequent

mutations and clonal selection in this new population of rapidly dividing cells may produce cells that are able to invade surrounding tissues in the body (Cooper 1992). Cancer cells can then be spread throughout the body by the circulatory system, an event called metastasis (Cooper 1992).

Cancer Treatment

The first option in cancer treatment is usually surgery. Surgery can be very effective in removing localized cancers but is not as useful in the treatment of cancers that have spread, since it is then difficult to remove all of the cancerous cells. Radiation therapy is utilized much like surgery for the treatment of localized cancers, but instead of removing the cells, it acts by damaging them so that they can no longer divide. Chemotherapy also kills or prevents cancer cells from replicating, and it can act on cells throughout the body. It is the most common therapy route utilized in cancer treatment because more than 70% of cancers have metastasized by the time they are diagnosed. Once a cancer has metastasized, localized treatments will not get rid of all of the cancer cells, and a more wide-scale treatment is required to stop the cancer cells that are circulating throughout the body. Chemotherapy is often used in combination with surgery or radiation therapy. (Cooper 1992).

A number of different drugs are available to treat cancer and usually are classified into four different groups: alkylating agents, antimetabolites, plant alkaloids, and antitumor antibiotics. Alkylating agents were the first cancer chemotherapy treatments available and are similar to mustard gas in composition. Nitrogen mustard and cyclophosphamide are two alkylating agents. They function by alkylating base pairs of the DNA, preventing it from uncoiling and therefore blocking DNA replication and transcription. Antimetabolites mimic the structure of DNA base pairs or other components of cellular metabolism, and function by blocking DNA synthesis or other metabolic functions. Methotrexate and 5-fluoruracil are commonly prescribed antimetabolites. The plant alkaloids inhibit mitosis through means other than DNA damage. Vincristine, vinblastine, and colchicine are plant-derived alkaloids that prevent the formation of the mitotic spindle. (Chemotherapy for Cancer 2002)

Many of the antitumor antibiotics function by directly binding to or intercalating into the DNA of tumor cells in order to prevent replication (Farmer 1985). Doxyrubicin (Adriamycin), bleomycin, mitomycin, and cisplatin are all antitumor antibiotics. The most widely used of these compounds is cisplatin [cis-diamminedichloroplatinum(II)] (Farmer 1985). The structure of this compound is shown in Figure 1.

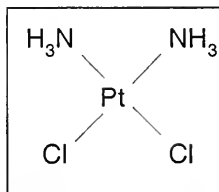


Figure 1. Chemical Structure of Cisplatin

Cisplatin Chemotherapy

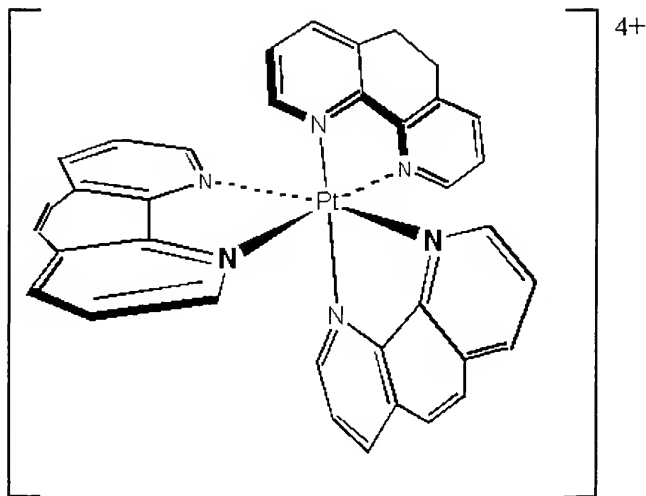
Cisplatin was first recognized for its anticancer capabilities in 1969 by Rosenberg and coworkers (Rosenburg 1980). Since that time much research has been devoted to it, and it was determined that the drug functions by forming interstrand and intrastrand crosslinks in DNA (Eastman 1985). Specifically, it has been found to form adducts between the N7 atoms of adjacent guanine rings (Eastman 1985). Cisplatin is a very useful drug because it is effective against a wide range of tumors, including both solid and disseminated tumors, both rapid and slow-growing tumors, and tumors resistant to other drug treatments (Farmer 1985). It does however, have a number of drawbacks. Cisplatin has can have considerable nephrotoxicity (which can be ameliorated by hydration therapy), and other rapidly dividing cells are affected by it (including hematopoietic cells in the bone marrow, epithelial cells, germ cells and hair forming cells) (Farmer 1985). One of the major impediments to patient quality of life is that both the drug and accompanying hydration must be administered intravenously (Farmer 1985). In addition,

not all tumors respond to treatment to cisplatin (Farmer 1985). Some tumors are initially resistant to it, and others can become resistant to it (Farmer 1985).

The New Pt and Pd Compounds

In the 1980s some researchers began to look at the interactions of octahedrally coordinated organometallic compounds with DNA. JK Barton did some particularly interesting investigations involving using transition metal compounds with known intercalative ligands as chiral probes that can cleave DNA (Barton 1986; Johann & Barton 1996). These studies included ruthenium and cobalt ions linked to phenanthroline ligands (Barton 1986). Although Barton compared the DNA binding of these compounds to that of cisplatin (1986), octahedrally coordinated platinum complexes were not included in these studies. This omission may have been due to the difficulty in synthesizing them (Granger et al. 2001).

Figure 2. The chemical structure of $[\text{Pt}(1,10\text{-phenanthroline})_3][\text{PF}_6]_4$, courtesy of Dr. Robert Granger (Sweet Briar College), from X-ray crystallography by Dr. Michal Sabat (University of Virginia).



In 1996, Dr. Robert Granger succeeded in synthesizing the first homoleptic water-soluble octahedral platinum (IV) complex, $[\text{Pt}(1,10\text{-phenanthroline})_3][\text{PF}_6]_4$. The structure of this compound is shown in figure 2. In the summer of 1997 Dr. Robin Davies tested this compound (along with several others

produced by Dr. Granger) on several cell lines. The new Pt(IV) and Pd(IV) compounds appeared to be very biologically active, and were specifically active on cancerous cell lines. Dr. Granger and his research team subsequently produced six additional Pt(IV) and Pd(IV) complexes of similar structure to be tested for cytotoxicity. All of these compounds are based on the transition metal center of either Pt(IV) or Pd(IV) with various known intercalative ligands attached. Dr. Granger's synthetic scheme proved to be sufficiently flexible to produce a number of compounds, and the ligands used included 5,6-dione-1,10-phenanthroline ("dione"), dipyridylphenazine ("dppz"), 2,2'-bipyridine ("bipy") and 1,10-phenanthroline ("phen") (see Fig. 3 for the structure of these ligands). Over the past several years, the original six compounds and others (with different combinations of the above ligands) produced by Dr. Granger have been tested for cytotoxicity against a variety of cells lines by Dr. Davies and a number of undergraduate researchers under her direction. Several compounds including Pt mono-dione [Pt(5,6-dione-1,10-phenanthroline)Cl₄] and Pd mono-dione [Pd(5,6-dione-1,10-phenanthroline)Cl₄] have shown clear selective toxicity for transformed cells over normal cells (Easterly 2000). Different combinations of ligands have been observed to have varying efficiencies in different cell lines, and the different metals have also proven more toxic to some cell lines than to others (Davies 1999).

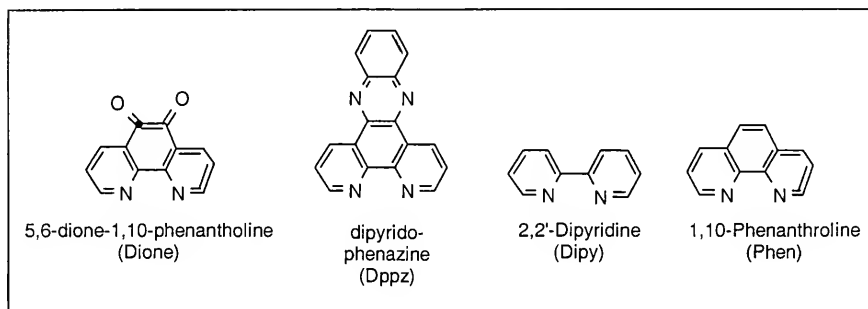


Figure 3. Chemical structures of the intercalating ligands focused on in this research.

Thousands of platinum compounds have been developed and researched because of the efficacy of cisplatin but less than thirty have reached clinical trials (Wong & Giandomencio 1999). Most of the

research efforts have focused on compounds which have (like cisplatin) a +2 oxidation state and a square planar geometry (Goddard et al. 1996). Beginning in the 1980s, a resurgence of research into platinum anticancer complexes occurred because of interest in platinum(IV) compounds (Wong & Giandomencio 1999). This was in part due to an interest in developing drugs for cisplatin resistant tumors, however Pt(IV) compounds have other useful characteristics. In general, Pt(IV) compounds are more stable and more soluble than Pt(II) compounds, allowing them to be administered orally (Giandomencio et al. 1994; Anderson et al. 1986). Oral administration could significantly improve quality of life for patients undergoing chemotherapy, possibly making it an outpatient treatment. In addition, several Pt(IV) complexes being tested for therapeutic use appear to be less nephrotoxic than the corresponding Pt(II) compounds (Giandomencio et al. 1994; Anderson et al. 1986).

Several platinum(IV) complexes, including iproplatin and tetraplatin, have undergone clinical trials (Wong & Giandomencio 1999). However, these compounds were abandoned due to their severe nephrotoxicity (Wong & Giandomencio 1999). JM-216 (*bis*-aceto-ammine-dichloro-cyclohexylamine platinum IV) is the first orally administered Pt(IV) compound to undergo clinical trials, which are currently in progress (Wong & Giandomencio 1999). Some evidence suggests that platinum(IV) compounds act as pro-drugs that get metabolized to platinum(II) complexes within cells (Anderson et al. 1986) but the mechanism of this reduction is not well understood (Wong & Giandomencio 1999).

The octahedrally coordinated compounds produced by Dr. Granger are completely different from the cisplatin analogues in size, charge, geometry, and presumed mode of action (Davies 1999). In addition, they differ from all other currently developed octahedrally coordinated compounds because of the size and complex organic structure of the ligands attached to the platinum ion. Dr. Granger's compounds more closely resemble the DNA probes researched by Barton than any other platinum anticancer complexes. However, cisplatin and its analogues are the closest related compounds available for comparison. Like cisplatin, the octahedrally coordinated compounds developed by Dr. Granger are cytotoxic *in vitro* as well as being preferentially toxic to tumor cells (Davies 1999).

In Vitro Cytotoxicity Testing and Anticancer Compounds

Since the 1950s and up until 1985 the screening of potential anticancer drugs was conducted *in vivo*, primarily utilizing mouse leukemia tumor models. In 1985, the National Cancer Institute began developing a new *in vitro* method for primary screening. In 1990, this *in vitro* operation screen was formally launched and has been the primary screening phase in anticancer drug development ever since. The screen incorporates sixty different human tumor cell lines including those derived from lung, colon, melanoma, renal, ovarian, brain and leukemia tumors. The standardized procedure involves first seeding a 96-well microtiter plate with the cells and incubating them for a 24 hour period before treatment. The cells are then treated with sample dilutions of the experimental compounds at concentrations of 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M and incubated for 48 hours. For the NCI screen, cells are assayed at the end of treatment using a sulforhodamine protein stain (SRB assay). (Boyd, 1997)

Although tetrazolium salt assays (using both MTT and XTT) were investigated for use as end-point assays, the NCI adopted the SRB assay because it was more suitable for large-scale screening. The SRB assay is more labor intensive, but it has a stable end-point (Boyd, 1997). The tetrazolium assays are time critical, but work well for small scale approaches such as those in this investigation.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay is a standard *in vitro* cytotoxicity assay (Guzzie, 1994). MTT is a yellow salt that is reduced by actively metabolizing cells to produce formazan, a blue dye (Guzzie, 1994). Succinate dehydrogenase, an enzyme in the TCA cycle, reduces the MTT to formazan (Guzzie, 1994). The amount of formazan can be quantitated using spectrometry at 540 nm, and is directly proportional to the number of viable cells (Fricker & Buckley 1996). For testing anticancer compounds on tumor cells, the MTT assay yields results equivalent to the SRB assay (Kratzke & Kramer 1996; Fricker & Buckley 1996).

The cytotoxicity experiments in this investigation were modeled on the procedures set forth by the NCI. It was determined in early studies that the experimental compounds were most effective at

concentrations of 10^{-5} M- 10^{-4} M (Davies 1999). Consequently those concentrations were used in this investigation. The MTT assay was chosen to assess cytotoxicity due to its ease of use. It is hoped that following the NCI procedures will produce data that suggest these compounds are promising enough to warrant further study. Ideally, compounds that target certain tumors, are more selective against transformed cells and that could be orally administered, could be developed from this research (Davies 1999).

Chemotherapy and Apoptosis

Although first described by Kerr et al. in 1972 (Kerr & Harmon 1991), the cellular phenomena of apoptosis did not gain much attention until the early 1990s, when there was a sudden great interest in it. One of the main reasons for this surge in research interest was the increasing molecular evidence of important links between cancer and cancer chemotherapies and apoptosis. As scientists began to uncover the molecular and genetic bases of cancer, the molecular mechanism of anticancer agents began to be more closely scrutinized. We now know much more about the intricately interconnected relationships between the mechanisms that cause tumor growth and those that cause tumor death, but many questions still remain unanswered, and the research continues. Apoptosis and its link to chemotherapy is of interest in this investigation because the mechanism of cell death produced by the novel cytotoxic agents investigated here has yet to be elucidated.

Apoptosis

Apoptosis is frequently referred to as “programmed cell death” because it involves the participation of the cell’s own internal mechanisms in its destruction. Apoptosis may occur under many normal physiological conditions, including during fetal development and in the elimination of aging or damaged cells. One of the characteristics of cancer cells is that they are immortal; though they may have detrimental genetic mutations, they do not undergo the process of apoptosis and instead accumulate to form growing tumors. Important new advances in the study of the mechanisms of death of tumor cells treated with chemotherapeutic agents indicate that these cells do undergo apoptosis (Debatin 2000).

Characteristics of apoptosis

Morphological

Apoptosis has a number of morphological and biochemical characteristics which distinguish it from necrosis. Necrosis was the first recognized mechanism of cell death, observed over a century ago (Barr & Tomei 1994). Necrosis usually occurs because of severe trauma to the cell from some external cause. Necrosis can be characterized by the swelling of the cell, the disruption of organelles (Wilson & Potten 1999) and the early loss of membrane integrity (Barr & Tomei 1994). In contrast, the most noticeable early morphological feature of apoptosis is cell shrinkage (Kerr & Harmon 1991) and blebbing of the cellular membrane. Other prominent characteristics of cells undergoing apoptosis are condensed chromatin and morphologically intact organelles (Wilson & Potten 1999).

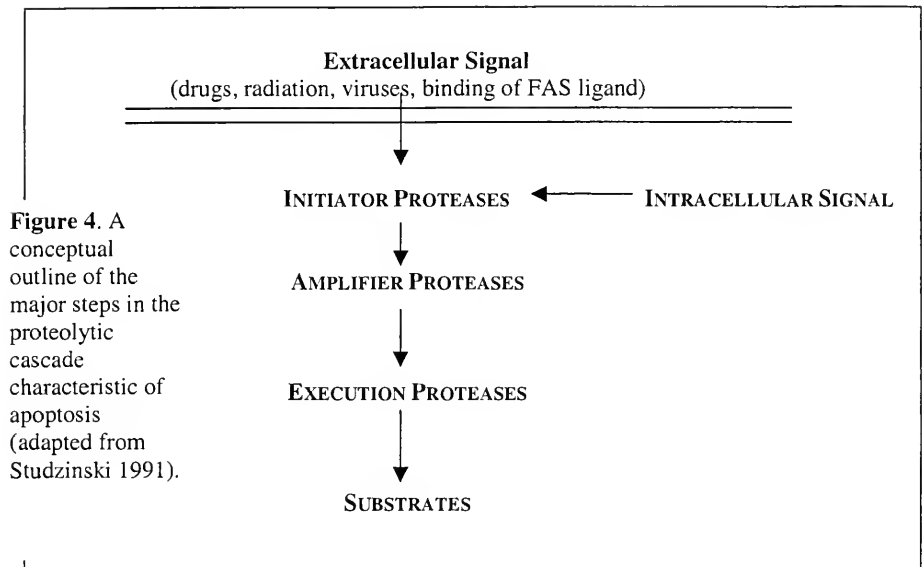
Apoptosis requires the expression of nuclear genes to produce the enzymes that ultimately break down the cellular membrane as well as the nuclear DNA. One of the most prominent distinguishing features of apoptosis is the breakdown of the DNA into nucleosomal fragments (50–300 kb), which produce a “ladder” when run through gel electrophoresis (Wilson & Potten 1999). The eventual breakdown of DNA during necrosis results in irregular chromatin clumping and produces random smears in gel electrophoresis. As a cell dies via apoptosis, the cellular contents are enclosed in vesicles created by the blebbing of the cellular membrane. *In vivo*, these apoptotic bodies are phagocytosed by adjacent cells (Kerr & Harmon 1991). If necrosis occurs in tissues, cellular contents are spilled into the intracellular spaces and inflammation results.

Studies have shown that the first of the morphological characteristics of apoptosis to become evident (when cells are treated with a cytotoxic substance) is the shrinkage of the cells. The shrinkage is followed by DNA fragmentation and fragmentation of the nucleus (Wilson & Potten 1999), and finally by loss of cellular membrane integrity as indicated by Trypan blue uptake (Barry et al. 1990). The exact timing of these events varies greatly depending on the cell types, the treatment used to induce apoptosis, and whether the experiment was *in vivo* or *in vitro*. In some cases, late stages of apoptosis are evident as

soon as 8 hours after cells are treated with a cytotoxic compound; in others, cell death is not complete until 1-2 days later (Barry et al. 1990).

Biochemical

It is thought that apoptosis the result of a very complex system of enzymes and cellular processes. A conceptual outline of the major steps in the biochemical pathway of apoptosis is shown in figure 4. The reason for the induction of apoptosis may be an internal factor (resulting from an event within the cell) or it may be external. Apoptotic signals include growth factors, hormones, radiation, hypothermia and various cytotoxic agents (Barr & Tomei 1994). Many proteases are involved in apoptosis. Proteases called caspases (because they cleave the carboxyl end of aspartate residues) appear to be essential to the apoptotic process (Blajeski and Kaufmann, 1999). The initial signal for apoptosis will put into action certain proteases, including several caspases and the serine protease granzyme B (Blajeski and Kaufmann, 1999), that will begin the apoptotic process. These initiator proteases will activate other proteases (the caspase cascade), which will serve to amplify the signal and will activate execution proteases (Studzinski, 1999). Execution proteases will carry out the various processes of breakdown of the cellular structures (Blajeski and Kaufmann, 1999). The mitochondria also appear to play a role in initiating the caspase cascade by contributing a signal that activates caspase 9 (an initiating protease) (Studzinski, 1999). One area of current research interest is in determining exactly how the first “signal” engages the initiator proteins of apoptosis.



Detection of apoptosis

Because there are several distinguishing morphological characteristics, there are a number of ways to determine if a cell is undergoing apoptosis. Nuclear counterstains (like thionin blue or haematoxylin) can be used on tissue sections or cells viewed under light microscopy in order to better visualize the condensed chromatin that stains particularly densely in cells which are undergoing apoptosis (Wilson & Potten 1999). The characteristic nuclear DNA fragments can be detected through terminal deoxynucleotide transferase mediated dUTP-biotin nick-end labeling (TUNEL) or DNA polymerase I-mediated *in situ* end-labeling (ISEL). The TUNEL technique takes advantage of the specific binding of terminal deoxynucleotidyl transferase to the 3'-OH ends of DNA (Gavrieli 1992). The transferase is used to incorporate a biotinylated deoxyuridine at the ends of DNA fragments (Gavrieli 1992). The experiments in this investigation utilized Roche's *In situ cell death detection kit*, which is a TUNEL assay that uses fluorescein-dUTP with results detectable by fluorescence microscopy. TUNEL labels

double-stranded DNA breaks, while ISEL labels single stranded breaks (Roche 2000). Consequently, TUNEL preferentially detects apoptosis over necrosis (Roche 2000).

Many other techniques can be useful in the detection of apoptosis and may involve examining the morphology or biochemical characteristics of the dying cells. Some of these techniques include gel electrophoresis (to detect the DNA “ladder,” track nuclear DNA fragmentation or compare the mitochondrial DNA to the nuclear DNA), assays of caspase activity, and a variety of biochemical assays which focus on specific steps of the apoptotic cascade (Wilson & Potten 1999). One other simple method of investigation used in this experiment is Trypan blue staining. Trypan blue is a stain that is excluded from live cells but will stain dead cells that have perforated membranes. Trypan blue staining can be used in cell death investigations to determine at what point membrane integrity is lost, which can help pinpoint the time frame of cell death in a particular system (Barry et al. 1990).

Apoptosis and Cancer

Apoptosis has been linked to cancer for several decades. Spontaneous apoptosis was observed in tumor cells as early as 1973 (Kerr et al. 1994). The growth of tumors frequently occurs because the tumor cells both increase their rate of growth and decrease their rate of death (Barr & Tomei 1994). In addition, it is apparent that these obviously damaged cells are not being removed from the population, which is one of the purposes of apoptosis. Scientists have known for quite some time that traditional cancer therapies such as irradiation, cytotoxic chemotherapy and hormone ablation increase the incidence of apoptosis in tumor cells (Kerr et al. 1994). More recent research has suggested that a variety of anticancer drugs do work by inducing apoptosis. Previously it was thought that the DNA cross-linking induced by many chemotherapeutic drugs simply prevented DNA synthesis. However, research on cisplatin indicated that cancer cells will die at drug concentrations that do not inhibit DNA synthesis (Barry et al. 1990). One model suggests that chemotherapeutic drugs work by generating damage signals where they are incorporated on the DNA and that it is these damage signals that ultimately act as signals for the initiator proteins.

Up to this point most cancer chemotherapy research has been conducted under the premise that if you get a high enough concentration of the drug to the cells, they will die. This has thus far not been a very useful assumption, having failed to produce any significant breakthroughs in treatment of drug resistant tumors in the past 30 years (Makin & Hickman 2000). Much more understanding has been gained about the molecular basis of apoptosis during this time, and it is now becoming clear that certain genes may be responsible for the induction of cell death by chemotherapeutic drugs. The two genes of current interest are the p53 tumor suppressor gene and the complex that produces the Bcl-2 protein. More research into the mechanisms of cell death induced by cytotoxic compounds may help in the future to “fine tune” cancer chemotherapy, creating drugs that better target transformed cells, and which are able to kill previously resistant cells.

Cisplatin and Apoptosis

Research into cell death resulting from treatment with cisplatin began in the 1980s and continues today. Many experimenters have observed that cisplatin causes the cell shrinkage and DNA digestion characteristic of apoptosis (Barry et al. 1990). Unfortunately, the mechanism by which cisplatin gets into cells is still not well understood (Gonzalez et al. 2001). Only about 1% of the cisplatin in the cell binds to the nuclear DNA, creating the characteristic adducts (Gonzalez et al. 2001). Other binding sites include any nucleophilic regions of the cell such as mitochondrial DNA, RNA, proteins, and the cytoskeleton. While it is suspected that the nuclear DNA lesions are the main trigger for apoptosis in cells treated with cisplatin, damage at these other binding sites may be involved. Some research has suggested that cells within the same populations may die by both necrosis and apoptosis (Gonzalez et al. 2001). It appears that the pathways of apoptosis induced by drugs may be very complex and could have a number of triggers and effectors (Debatin 2000). Some research suggests that the p53 gene, which has been shown to affect cell sensitivity to cisplatin, may be the major contributor to initiating apoptosis but this also is not well understood (Debatin 2000). Overall, the way cisplatin triggers apoptosis is still unclear (Gonzalez et al. 2001). Thus, research into apoptosis induced by cisplatin and by other anti-

tumor drugs could provide the necessary understanding and might then have a significant impact on the practice of cancer chemotherapy.

Purpose:

The purpose of this investigation is to learn more about the biological activity of the newly synthesized Pt and Pd complexes. This investigation has several parts including comparing the relative sensitivities of several cell lines to some of the compounds, determining where the compounds localize within cells, determining the mechanism of cell death induced by these complexes and gaining a better understanding of this mechanism.

Materials and Methods:

Procedure for Cytotoxicity Testing

Cell Culture

Three cell lines were used in this investigation: HTB-125, HTB-126 and CCD-27Sk. These cell lines were obtained from the American Type Culture collection. The HTB-125 and HTB-126 are matched human cell lines of normal and transformed (respectively) fibroblast cells obtained from the breast tissue biopsy of a 74 year old, Caucasian female. The HTB-125 line is from normal breast tissue that was peripheral to an infiltrating ductal carcinoma (from which the HTB-126 line was derived). The CCD-27Sk cells are normal human skin fibroblasts derived from the neonatal tissue of a Black male at 23 weeks gestation who died from complications following a premature birth.

The cells were cultured in standard tissue culture plastic flasks in a VWR model 2475 B incubator with the temperature maintained at 37° C, relative humidity of 88% and CO₂ concentration of 5%. All of the lines were grown in 90 % DMEM (Dulbecco's Modified Eagle Medium with GlutaMAX™, high glucose, 110mg/L sodium pyruvate, and pyroxidine-HCL) supplemented with 10% fetal bovine serum, 30 ng/ml human epidermal growth factor (EGF), and 10 µg/ml bovine insulin. All media components were obtained from Gibco/Life Technologies. Later in the investigation, the HTB-126 cells were grown in medium without the human EGF, with no ill effects or changes in culture characteristics. Cells were

subcultured at 1:2, 1:3, and 1:6 ratios using a 0.25% Trypsin/ 1 μ M EDTA solution diluted at a 1:2

ratio in phosphate buffered saline solution (PBS).

Preparation of Microplates

Upon reaching confluency, cells were trypsinized to detach them from the subculture flasks and then transferred to ninety-six well microplates. Each T-25 flask yielded sufficient cells for two microplates and each T-75 flask yielded sufficient cells for 6 microplates. To each well of the microplate 200 μ L of cell suspension was added, and the microplates were replaced in the incubator for 24-72 hours prior to treatment with the experimental compounds.

Experimental Compounds

Four different transition metal compounds were investigated: Pt(dione)Cl₄, Pd(dione)Cl₄, Pd(dppz)Cl₄, and Pd(dppz)₂Cl₂ (see fig. 3 for the structure of the ligands). These compounds be referred to throughout the rest of this paper as Pt mono-dione, Pd mono-dione, Pd mono-dppz and Pd bis-dppz, respectively. A 10⁻³ M dilution in DMSO (dimethyl sulfoxide, Sigma) was made for each compound and kept frozen to be used as a stock solution for each trial. Prior to each trial, the stock solution was thawed and a 10⁻⁴ M dilution was made in culture medium. As a control, a solution of 10% DMSO in culture medium (DMEM) was also made. Wells in the microplate were treated with 20 μ L of the solutions; one column of untreated wells was placed in between each three-column block of treatments. Each 96-well microplate therefore contained 3 columns (24 wells) of untreated cells, 3 columns of cells treated with the 10% DMSO solution (for a final concentration of 1% DMSO), three columns of cells treated with one compound, and 3 columns of cells treated with the second compound. For routine cytotoxicity assays the final concentration of the experimental compound in each well was 10⁻⁵ M.

Cytotoxicity (MTT) Assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, from Sigma) was used to assay the cytotoxicity of the Pd and Pt compounds. MTT is a terazolium salt that is reduced by metabolically active cells to produce an insoluble formazan dye (Roche 1999). The purple formazan

crystals produced by the cells are solubilized with DMSO and the absorbance read by a Tecan Spectra Shell spectrophotometer. The amount of formazan produced corresponds directly to the number of metabolically active cells present (Roche 1999).

After treatment with the Pd or Pt compounds the microplates were incubated for 48 hours, then 20 μ L of MTT dissolved in PBS (5mg/mL) was added to each well and the microplates were incubated for an additional 2-4 hours. The medium was then removed from the plates and 100 μ L of DMSO was added to each well to disrupt cell membranes and dissolve the blue formazan. The absorbance at 540 nm was then determined using the plate reader.

Statistical Analysis

For each plate the percent survival of the treated cells was calculated by taking the average absorbencies of all the wells in the treatment group and dividing it by the average of all the wells treated with 10% DMSO, then multiplying by one hundred. Further analysis was conducted for each microplate by comparing all the wells in each treatment group via a 1-way ANOVA test and the Fischer's Protected LSD post-hoc test, using the SuperANOVA software program from Abacus Concepts. The Fischer's Protected LSD post-hoc test was chosen because it is a less conservative test. A significance level of 0.05 was chosen.

Procedure for Investigating Mechanisms of Cells Death

Slide Preparation

In preparation for the TUNEL assay, cells were subcultured on glass Lab-Tek™ Chamber Slides™ cell/tissue culture slides. There were two chambers on each slide and each was treated as a separate sample, which held 1mL of medium.

Experimental Compounds

The cytotoxic compounds used in this study were Pd mono-dione and Pt mono-dione obtained from Dr. Robert Granger. The compounds were dissolved in reagent grade DMSO at a 10⁻³ M concentration, then diluted to a concentration of 10⁻⁴ M in the same DMEM solution used to culture the cells. Prior to

the TUNEL assay, confluent cells grown on culture slides were treated with enough 10^{-4} M solution to yield a final concentration of 10^{-5} M when combined with the media already in the chambers. The slides were then incubated for 20-30 hours. After incubation, the medium was removed and the chamber was separated from the slide, leaving the adherent cell sample intact.

TUNEL assay

The *In situ cell death detection kit, Fluorescein* from Roche Biochemical was used to perform this TUNEL assay. The adherent cell samples were first air-dried for 10-15 minutes after the medium was removed, then fixed with a freshly prepared 4% paraformaldehyde solution at pH 7.4 for a period of one hour at room temperature. After fixation, the cells were rinsed with PBS and incubated in a solution of 0.1% Triton X-100 and 0.1% sodium citrate for two minutes on ice in order to permeabilize the cell membranes. After two minutes, the slides were rinsed twice with PBS and TUNEL reaction mixture was pipetted onto each sample. TUNEL reaction mixture was prepared by adding terminal transferase solution to the label solution (according to the manufacturers directions). The samples were then covered with a coverslip and incubated in a humidified chamber for one hour in the dark.

Negative controls were prepared by treating fixed and permeabilized cells with labeling solution (without terminal transferase) then incubating for one hour in the humidified chamber. Positive controls were prepared by treating the fixed and permeabilized cells with DNase I (grade I: 1mg/ml in 50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mg/ml BSA) for 10 minutes at room temperature (25° C), then adding the TUNEL reaction mixture and incubating as above. After incubation, cells were covered with an anti-fade mountant and a cover slip, then viewed by fluorescence microscopy at an excitation wavelength of 450-500 nm and detection wavelength of 515-565. Photomicrographs were taken using 800 speed film, and developed by a commercial photo lab. Additional photomicrographs were taken with a digital camera in Daniel Burke's lab, Dept. of Biochemistry and Molecular Genetics, University of Virginia.

Trypan Blue Assay

For the Trypan blue assay, HTB-126 cells were grown on culture slides until confluent, as for the TUNEL assay. Upon reaching confluency, the cells were treated with 10^{-5} M Pt mono-dione in DMEM. Several drops of sterile Trypan blue were added to each well at intervals of 24, 27, 30, and 36 hours after treatment and were allowed to stand for several minutes before the culture wells were removed and the slides were covered with coverslips and viewed under the light microscope. Photomicrographs were taken using 400 speed Kodak film, and processed in a commercial photo lab.

Gel Electrophoresis

Agarose (Sigma) was used to prepare a 1.5% agarose gel, containing 0.5 $\mu\text{g/ml}$ ethidium bromide (Sigma). DNA that was extracted from the HTB-126 cells for analysis using atomic absorption spectrometry (procedure described below) was used for this experiment. The HTB-126 DNA used included 10 μg and 15 μg samples of DNA from cells that had not been treated with compounds (the control), cells that had been treated with 10^{-4} M Pd mono-dione, and cells that had been treated with 10^{-5} M Pd mono-dione. The marker DNAs used were bacteriophage lambda DNA digested with Hind III obtained from New England BioLabs, and a 1 Kb DNA ladder obtained from Gibco/Life Technologies. High molecular weight DNA isolated from AHH-1 human lymphoblast cells (ATCC No. CRL-8146) by Dr. Robin Davies was used as an additional control. Samples were loaded with 12 μl sample buffer (30 % glycerol and 0.25 % bromophenol blue). The electrophoresis was run in 1x TBE (0.9 M Tris-borate, 0.002 M EDTA, pH 8.0) at 80 volts for approximately 6 hours. Polaroid photos were taken on a UV light box (Fotodyne system) using a shutter speed of 1/8 sec and an F-stop of 4.5.

Procedure for Investigating Localization of Cytotoxic Compounds

Cell Culture

HTB-126 cells were cultured as described above in 150 cm^2 peel-off tissue culture flasks. In preparation for fractionation or DNA extraction, cells were collected by removing growth media, rinsing twice with PBS, and removing from the flask surface by scraping and rinsing with PBS. The cells were

then centrifuged at $\sim 400 \times g$ for 5 minutes. The cells were resuspended in PBS and centrifuged at $\sim 400 \times g$ for 5 minutes if destined for DNA extraction or at $500 \times g$ for 2-3 minutes if destined for fractionation.

Experimental Compounds

The compound used during this section of the investigation was Pd mono-dione prepared as above. The treatment concentrations were 10^{-4} M and 10^{-5} M. Cells treated with 10^{-4} M Pd mono-dione were exposed to the compound for approximately one hour before being collected. Cells treated with 10^{-5} M Pd mono-dione were exposed to the compound for approximately ten hours before being collected. Treatment times were determined by a time course experiment using methylene blue to determine the average time before nucleus disintegration at each concentration, and by observation of treated cells by phase contrast microscopy. Control cells for this experiment were treated with the DMEM media used to culture the cells plus 10% DMSO, for a final concentration of 1% DMSO.

Nucleus and Cytoplasm Fractionation

Nuclear and cytoplasmic fractions of the cells were obtained using the *NE-PER™ Nuclear and Cytoplasm Extraction Reagents Kit*, from Pierce Chemical Co. The manufacturer's instructions were followed to extract the cytoplasm and isolate the nuclei. Each extraction was performed on approximately 4×10^6 cells. The final nuclear extraction step was skipped in order to isolate whole nuclei. Fractions were stored at -80°C .

DNA Extraction

DNA extraction was performed using a *DNA Extraction Kit (Non-organic)* from Intergen Co, following the manufacturer's instructions. Again, each extraction was performed on approximately 4×10^6 cells. Briefly, cells were lysed with "Wash Solution", pelleted and resuspended in "Suspension Buffer I." DNA was deproteinated with "Protein Digesting Enzyme" for two hours at 50°C or overnight at 37°C . The protein was precipitated with "Protein Precipitating Agent" and the proteins were pelleted by centrifugation. The DNA was precipitated from the supernatant with absolute ethanol and then

resuspended in "Suspension Buffer II" in a 55° C water bath overnight. The DNA concentration of each sample was calculated from the absorption at 260 nm.

Acid Preparation of Whole Cell, Cell Fraction, and DNA samples for Atomic Absorption Spectrometry

The samples prepared for analysis with atomic absorption spectrometry included: whole cells treated with 10^{-4} M Pd mono-dione, whole cells treated with 10^{-5} M Pd mono-dione, a control sample of whole cells; cytoplasm and nuclei fractions of control cells, cells treated with 10^{-4} M Pd mono-dione, and cells treated with 10^{-5} M Pd mono-dione; DNA extracted from control cells, cells treated with 10^{-4} M Pd mono-dione, and cells treated with 10^{-5} M Pd mono-dione.

Concentrated nitric acid (HNO_3 , 15M) was used to digest all of the samples. After adding the acid the whole cell samples were boiled for 10 minutes until solutions appeared clear. Samples were then diluted to 5% HNO_3 using ddH_2O . When refrigerated, white precipitate formed in samples containing nuclei or cytoplasm fractions, but could be removed by heating and vortexing. This acid preparation procedure is a standard method of preparing biological samples for atomic absorption spectrometry (Wei et al. 1999).

Atomic Absorption Spectrometry

Atomic absorption spectrometry was performed on all samples using an atomic absorption spectrometer (Instrumentation Laboratories) at Lynchburg College under the direction of Dr. Priscilla Gannicott. A 247.6 nm hollow cathode Pd lamp with an air acetylene flame (oxidizing fuel, lean, blue) was used, with a current of 20 mA to the hollow cathode lamp and 700 volts to the photomultiplier tube. Dilutions of 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M of Pd mono-dione in 5% HNO_3 were prepared and analyzed in an attempt to create a calibration curve.

Results:

Comparisons of Cytotoxicity

The percent survival for each group of treated cells was calculated by dividing the treatment group average absorbance by the average absorbance for the DMSO control group on the same plate and then

multiplying by 100. These percent survivals are presented in figures 5-10. Figures 5-7 are graphs comparing percent survivals of cells treated with Pt mono-dione or Pd mono-dione. Figures 8-10 show comparisons of the percent survivals of cells treated with Pd mono-dppz and Pd bis-dppz.

Figure 5 shows a comparison of the percent survivals of HTB-125 (normal) cells treated with Pt mono-dione and Pd mono-dione. The data from two microplates is shown on this figure. Both plates show similar percent survivals for both compounds, and the cells treated with Pd mono-dione have a higher percent survival than those treated with Pt mono-dione.

Figure 5. Percent Survival of HTB-125 cells treated with Pt mono-dione and Pd mono-dione

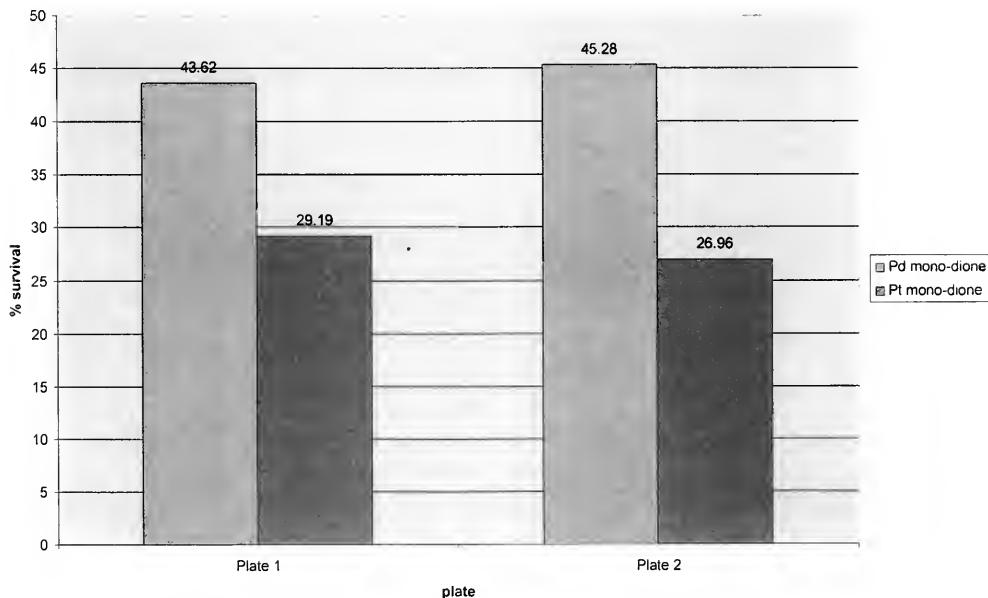


Figure 6 is a comparison of the percent survivals of CCD-27Sk (normal) cells treated with the same compounds. The percent survivals from two plates are shown. This graph shows that the percent

survivals for cells treated with Pd mono-dione was lower on both plates than the percent survivals for the cells treated with Pt mono-dione. However, the percentages for the two different metals are very similar on both plates.

Figure 7 is a comparison of the percent survivals of HTB-126 (transformed) cells treated with Pd mono-dione or Pt mono-dione. The data from eleven plates is shown on this graph. These plates were labeled with a letter and a number; the letter indicates which flask the cells on that plate came from and the number indicates the order in which the cells were plated. This labeling system was developed in order to better compare the results, since cells that came from the same flask could be expected to behave similarly to each other but might behave differently from cells from other flasks (all the plates of non-transformed cells that will be discussed came from different flasks). In all eleven plates the cells treated with Pd mono-dione had a higher percent survival than the cells treated with Pt mono-dione. However, the amount by which these survival rates differ varies slightly between plates from the same flask and even more between plates from different flasks.

Figure 6. Percent Survival of CCD-27Sk cells treated with Pt mono-dione and Pd mono-dione

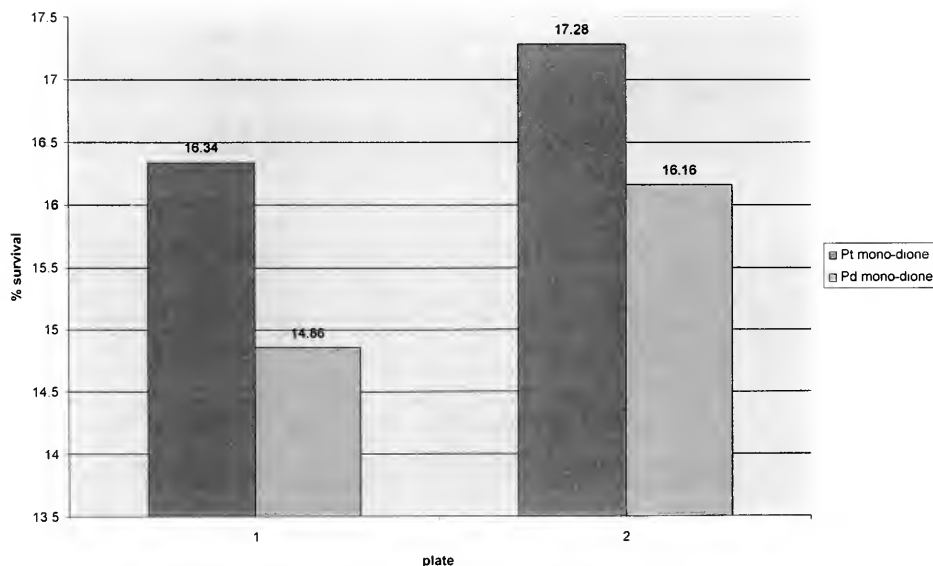


Figure 7. Percent survival of HTB-126 cells treated with Pt mono dione or Pd mono dione

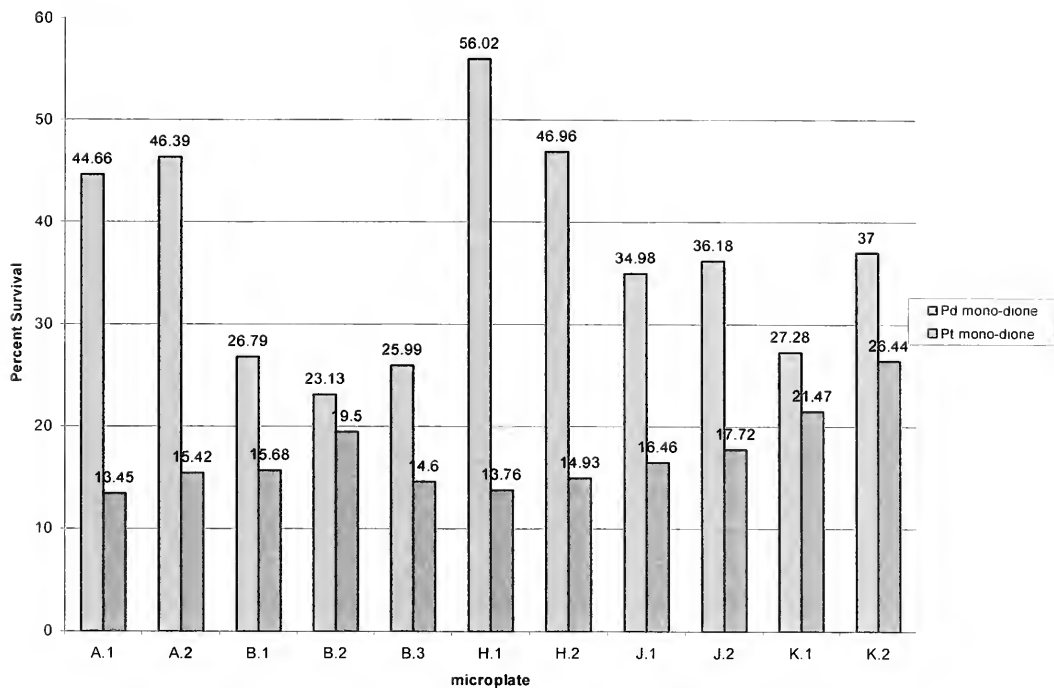


Figure 8. Percent Survival of HTB-125 cells treated with Pd mono-dppz and Pd bis-dppz

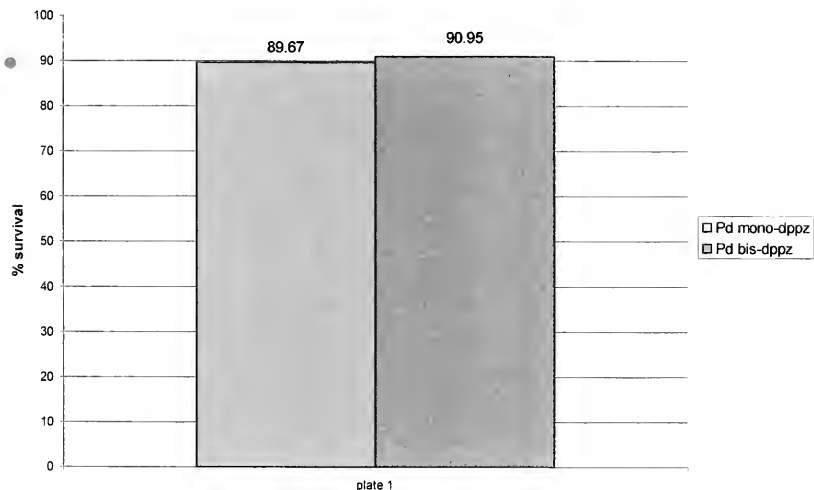
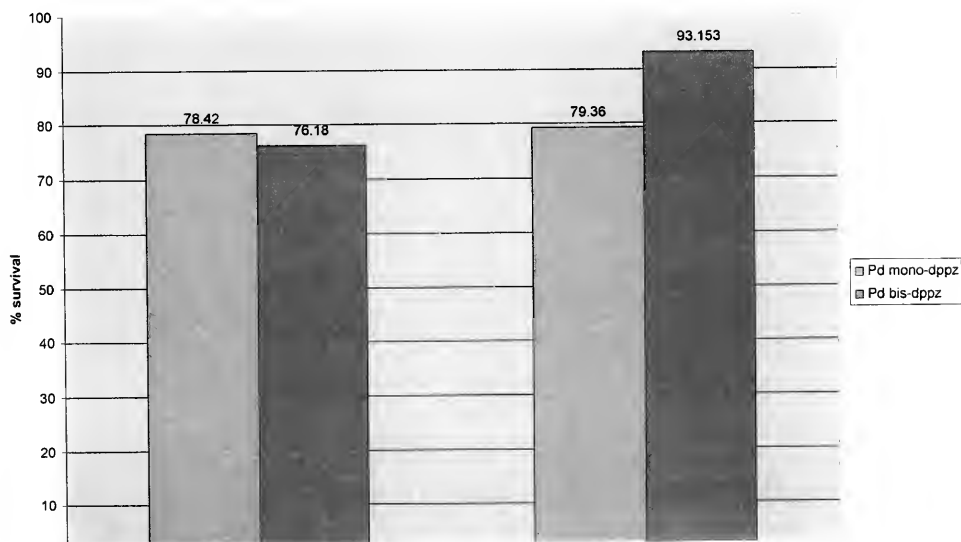


Figure 8 shows the percent survival data from one microplate of HTB-125 cells treated with Pd mono-dppz or Pd bis-dppz. Although the scale of the chart makes it appear that there is a significant difference between the percent survivals of the cells treated with the two compounds, the Pd bis-dppz was only actually slightly more effective in killing cells. Figure 9 shows the data from two microplates of CCD-27Sk cells treated with Pd mono-dppz or Pd bis-dppz. The first plate shows a slightly higher percent survival for the cells treated with Pd mono-dppz, while the second plate shows a much higher percent survival for the cells treated with Pd bis-dppz.

Figure 10 shows the percent survival data from seven microplates of HTB-126 cells treated with Pd mono-dppz or Pd bis-dppz. These seven microplates came from three different flasks. The first two plates (from flask C) have similar percent survivals, with the Pd mono-dppz killing more than twice as many cells as the Pd bis-dppz. The next three plates (from flask A) also show a lower percent survival for cells treated with the Pd mono-dppz, but the difference between the percent survivals of the two compounds is not as great as the difference in the first two plates. The last two sets of columns on this graph show the percent survivals for two plates from the same flask. The first of these (G.1) shows a

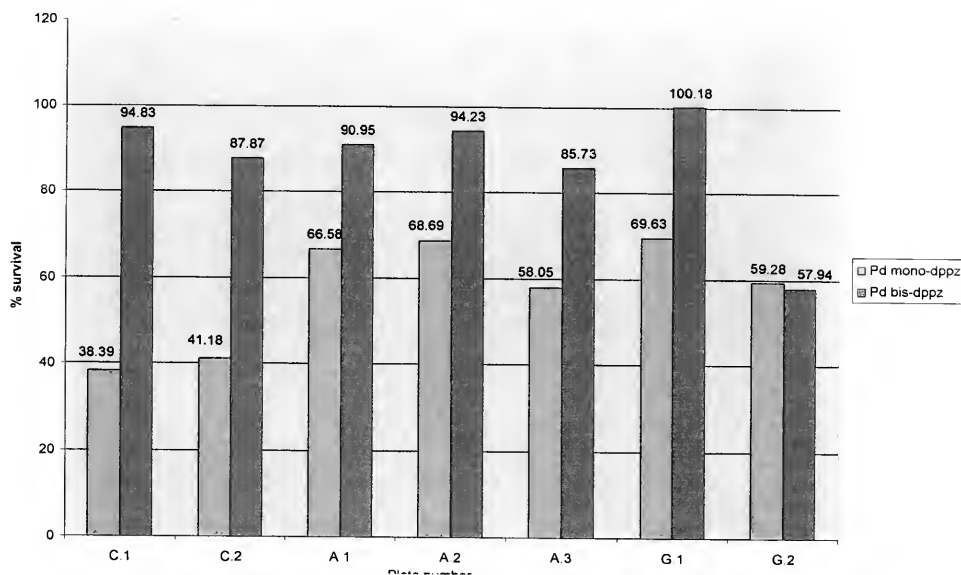
Figure 9. Percent Survival of CCD-27Sk cells treated with Pd mono-dppz and Pd bis-dppz



lower percent survival for cells treated with Pd mono-dppz, concurrent with the rest of the data in this particular trial. The graph of the data from plate G.2 however, shows a very small difference between the two treatment groups and shows the cells treated with Pd mono-dppz as having a higher survival rate.

Figure 10.

Percent survival of HTB-126 cells treated with Pd mono-dppz and Pd bis-dppz



Figures 11-16 are photographs of HTB-125 cells either untreated or treated with Pd mono-dione, Pt mono-dione or Pd mono-dppz. All of the cells were from the same flask and grown on the same culture slide, then photographed after being exposed to the compounds for 48 hours. Figure 11 is a photograph of HTB-125 cells that weren't treated with any compounds. These cells are regularly spaced, rather elongated in shape and all oriented in one direction in parallel arrays. They look characteristically like normal fibroblasts.

Figure 12 is a photograph of HTB-125 cells treated with Pd mono-dione. The cells in this picture are either dying or in a state of crisis. In contrast to the regular conformations of the untreated cells,

these cells are irregularly shaped and appear more spread out. The slide is littered with what appears to be pieces of cellular material which are probably bits of cytoskeleton and plasma membrane representing sites where the cells were attached to the slide surface before they were treated. Figure 13 is a photograph of HTB-125 cells treated with Pt mono-dione. These cells are clearly dead and have mostly detached from the slide, appearing as small dark circles. These cells appear much smaller in volume

Figure 14 is a photograph at the scanning (~140x) magnification of HTB-125 cells treated with Pd mono-dppz. These cells appear relatively unaffected by the compound, looking only slightly more irregular than untreated cells. The cells are slightly less parallel, and there is some material outside of the cells. Figures 15 and 16 are also HTB-125 cells treated with Pd mono-dppz, this time photographed at ~352x magnification. The most noticeable feature of these photographs are short rod-shaped crystals present between the cells. These crystals are most likely un-dissolved Pd mono-dppz.

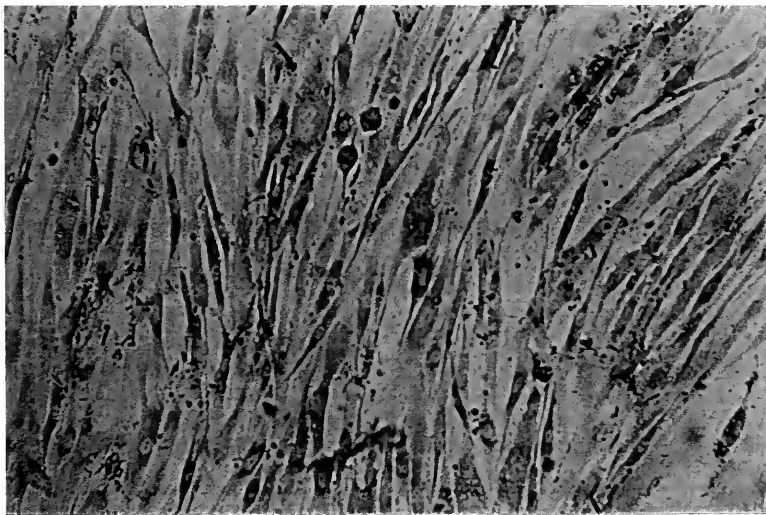


Figure 11. HTB-125 cells untreated magnified approx. 140x.



Figure 12. HTB-125 cells treated with Pd mono-dione magnified approx. 140x.

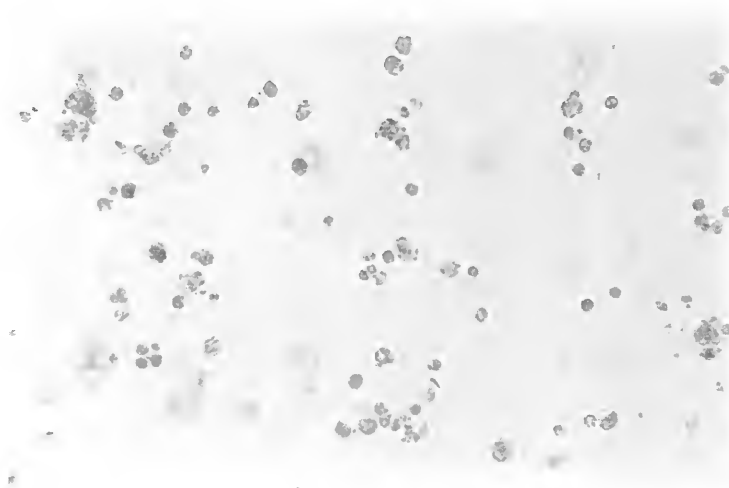


Figure 13. HTB-125 cells treated with Pt mono-dione magnified approx. 140x.



Figure 14. HTB-125 cells treated with Pd mono-dppz magnified approx. 140x.

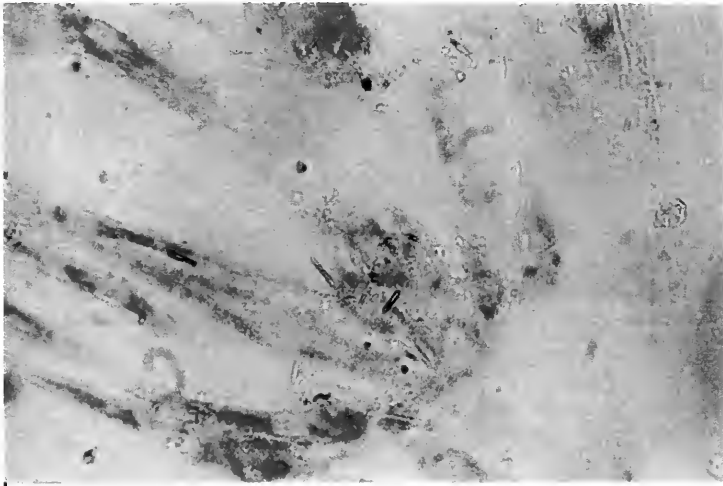


Figure 15. HTB-125 cells treated with Pd mono-dppz magnified approx. 352x.



Figure 16. HTB-125 cells treated with Pd mono-dppz magnified approx. 352x.

Statistical Data Analysis

The following tables show the p-value and the significance of the means of absorbance compared with the type of cell treatment, as determined using Fischer's Protected Least Significance Difference test.

Table 1. HTB-125 cells treated with Pd mono-dione or Pt mono-dione

comparison	Plate 1	significance?	Plate 2	significance?
	p-value		p-value	
Pd mono-dione vs. Pt mono-dione	0.0001	S	0.0001	S
Pd mono-dione vs. DMSO	0.0001	S	0.0001	S
Pd mono-dione vs. cells only	0.0001	S	0.0001	S
Pt mono-dione vs. DMSO	0.0001	S	0.0001	S
Pt mono-dione vs. cells only	0.0001	S	0.0001	S
DMSO vs. cells only	0.0016	S	0.8069	NS

Table 2. CCD-27Sk cells treated with Pt mono-dione or Pd mono-dione

comparison	Plate 1	significance?	Plate 2	significance?
	p-value		p-value	
Pd mono-dione vs.. Pt mono-dione	0.7799	NS	0.6736	NS
Pd mono-dione vs.. DMSO	0.0001	S	0.0001	S
Pd mono-dione vs. cells only	0.0001	S	0.0001	S
Pt mono-dione vs. DMSO	0.0001	S	0.0001	S
Pt mono-dione vs. cells only	0.0001	S	0.0001	S
DMSO vs. cells only	0.0001	S	0.0001	S

Table 3. HTB-126 cells treated with Pt mono-dione or Pd mono-dione

comparison	Plate A.1		Plate A.2			
	p-value	significance?	p-value	significance?		
Pd mono-dione vs. Pt mono-dione	0.0001	S	0.0001	S		
Pd mono-dione vs. DMSO	0.0001	S	0.0001	S		
Pd mono-dione vs. cells only	0.0001	S	0.0001	S		
Pt mono-dione vs. DMSO	0.0001	S	0.0001	S		
Pt mono-dione vs. cells only	0.0001	S	0.0001	S		
DMSO vs. cells only	0.5369	NS	0.0362	S		
comparison	Plate B.1		Plate B.2		Plate B.3	
	p-value	significance?	p-value	significance?	p-value	significance?
Pd mono-dione vs. Pt mono-dione	0.0001	S	0.2519	NS	0.0027	S
Pd mono-dione vs. DMSO	0.0001	S	0.0001	S	0.0001	S
Pd mono-dione vs. cells only	0.0001	S	0.0001	S	0.0001	S
Pt mono-dione vs. DMSO	0.0001	S	0.0001	S	0.0001	S
Pt mono-dione vs. cells only	0.0001	S	0.0001	S	0.0001	S
DMSO vs. cells only	0.2295	NS	0.0463	S	0.2498	NS
comparison	Plate H.1		Plate H.2			
	p-value	significance?	p-value	significance?		
Pd mono-dione vs. Pt mono-dione	0.0001	S	0.0001	S		
Pd mono-dione vs. DMSO	0.0001	S	0.0001	S		
Pd mono-dione vs. cells only	0.0001	S	0.0001	S		
Pt mono-dione vs. DMSO	0.0001	S	0.0001	S		
Pt mono-dione vs. cells only	0.0001	S	0.0001	S		
DMSO vs. cells only	0.0001	S	0.9112	NS		
comparison	Plate K.1		Plate K.2			
	p-value	significance?	p-value	significance?		
Pd mono-dione vs. Pt mono-dione	0.0001	S	0.0001	S		
Pd mono-dione vs. DMSO	0.0001	S	0.0001	S		
Pd mono-dione vs. cells only	0.0001	S	0.0001	S		
Pt mono-dione vs. DMSO	0.0001	S	0.0001	S		
Pt mono-dione vs. cells only	0.0001	S	0.0001	S		
DMSO vs. cells only	0.0001	S	0.0001	S		
comparison	Plate J.1		Plate J.2			
	p-value	significance?	p-value	significance?		
Pd mono-dione vs. Pt mono-dione	0.0001	S	0.0001	S		
Pd mono-dione vs. DMSO	0.0001	S	0.0001	S		
Pd mono-dione vs. cells only	0.0001	S	0.0001	S		
Pt mono-dione vs. DMSO	0.0001	S	0.0001	S		
Pt mono-dione vs. cells only	0.0001	S	0.0001	S		
DMSO vs. cells only	0.0044	S	0.0001	S		

Table 4. HTB-125 cells treated with Pd mono-dppz or Pd bis-dppz

comparison	Plate 1	
	p-value	significance?
Pd mono-dppz vs Pd bis-dppz	0.6195	NS
Pd mono-dppz vs DMSO	0.0001	S
Pd mono-dppz vs cells only	0.0001	S
Pd bis-dppz vs DMSO	0.0006	S
Pd bis-dppz vs cells only	0.0001	S
DMSO vs cells only	0.0121	S

Tables 1-6 show the statistical values obtained when the absorbance values for each set of treatment groups on each plate were compared using Fischer's Protected Least Significance Difference test. A p-value of 0.05 was set as the significance level. The "significance?" column indicates whether or not the p-value is significant, "S" means the value is significant, "NS" means the value is not significant. Table 1 shows the p-values for two plates of HTB-125 cells treated with Pd mono-dione or Pt mono-dione, and whether these were significant. For this trial, both plates showed a significant difference in absorbance between the wells treated with Pt mono-dione and the wells treated with Pd mono-dione. In addition, both plates showed that there was a significant difference between the absorbances of the wells treated with either the Pd mono-dione or Pt mono-dione and the wells in the control groups of either un-treated cells or cells treated with 1 % DMSO.

Table 5. CCD-27Sk cells treated with Pd mono-dppz or Pd bis-dppz

comparison	Plate 1		Plate 2	
	p-value	significance?	p-value	significance?
Pd mono-dppz vs. Pd bis-dppz	0.4511	NS	0.0018	S
Pd mono-dppz vs. DMSO	0.0001	S	0.0001	S
Pd mono-dppz vs. cells only	0.0001	S	0.0001	S
Pd bis-dppz vs. DMSO	0.0001	S	0.1212	NS
Pd bis-dppz vs. cells only	0.0001	S	0.0001	S
DMSO vs. cells only	0.0001	S	0.0001	S

Table 6. HTB-126 cells treated with Pd mono-dppz or Pd bis-dppz

comparison	Plate C.1		Plate C.2			
	p-value	significance?	p-value	significance?		
Pd mono-dppz vs Pd bis-dppz	0.0001	S	0.0001	S		
Pd mono-dppz vs DMSO	0.0001	S	0.0001	S		
Pd mono-dppz vs cells only	0.0001	S	0.0001	S		
Pd bis-dppz vs DMSO	0.1664	NS	0.0288	S		
Pd bis-dppz vs cells only	0.0068	S	0.0112	S		
DMSO vs cells only	0.1732	NS	0.7147	NS		
comparison	Plate A.1		Plate A.2		Plate A.3	
	p-value	significance?	p-value	significance?	p-value	significance?
Pd mono-dppz vs. Pd bis-dppz	0.0001	S	0.0001	S	0.0001	S
Pd mono-dppz vs. DMSO	0.0001	S	0.0001	S	0.0001	S
Pd mono-dppz vs. cells only	0.0001	S	0.0001	S	0.0001	S
Pd bis-dppz vs. DMSO	0.0277	S	0.1313	NS	0.0001	S
Pd bis-dppz vs. cells only	0.0001	S	0.0025	S	0.0001	S
DMSO vs. cells only	0.0074	S	0.115	NS	0.3467	NS
comparison	Plate G.1		Plate G.2			
	p-value	significance?	p-value	significance?		
Pd mono-dppz vs. Pd bis-dppz	0.0001	S	0.7819	NS		
Pd mono-dppz vs. DMSO	0.0001	S	0.0001	S		
Pd mono-dppz vs. cells only	0.0001	S	0.0001	S		
Pd bis-dppz vs. DMSO	0.9163	NS	0.0001	S		
Pd bis-dppz vs. cells only	0.0719	NS	0.0001	S		
DMSO vs. cells only	0.0572	NS	0.3438	NS		

Table 2 shows the statistical analysis (p-values and significance) for CCD-27Sk cells treated with Pt mono-dione or Pd mono-dione solutions. For this cell line, there was no significant difference in either plate between the absorbances of wells treated with Pt mono-dione and those treated with Pd mono-dione. There is a significant difference in both plates between the absorbances of those wells treated with the compounds and the control wells that contained either un-treated cells or cells treated with 1% DMSO.

Table 3 shows the statistical values for eleven plates of HTB-126 cells treated with the Pt mono-dione or Pd mono-dione solutions. Every plate except B.2 showed that there was a significant difference between the absorbances of the wells treated with Pt mono-dione and those treated with Pd mono-dione. In addition, all eleven plates showed a significant difference between the absorbances of the wells

treated with either the Pt mono-dione or Pd mono-dione solutions and the control wells which were either untreated or treated with 1% DMSO.

Table 4 shows the statistical data for one plate of HTB-125 cells treated with Pd mono-dppz and Pd bis-dppz solutions. This plate showed no significant difference between the absorbances of the wells treated with the Pd mono-dppz and the wells treated with Pd bis-dppz. There was however, a significant difference between the Pd mono-dppz treated wells and the control wells, and also a significant difference between the wells treated with Pd bis-dppz and the control wells.

Table 5 contains that statistical data for two plates of CCD-27SK cells treated with Pd mono-dppz and Pd bis-dppz solutions. The first plate showed a significant difference between the absorbances of the wells treated with the Pd mono-dppz solution and the wells treated with Pd bis-dppz solution. This plate also had significant differences between both of the Pd compounds and the control groups. The second plate in this trial showed a significant difference between the Pd mono-dppz and Pd bis-dppz, but no significant difference between the wells treated with Pd bis-dppz and the wells treated with 1% DMSO.

Table 6 contains the statistical values for seven microplates of HTB-126 cells treated with Pd mono-dppz or Pd bis-dppz solutions. Six of these plates show that there is a significant difference between the absorbances of wells treated with Pd mono-dppz and those treated with Pd bis-dppz. The seventh plate (G.2) is incongruent and shows no significant difference. Plates C.1, A.2 and G.1 showed no significant difference between the wells treated with Pd bis-dppz and those treated with 1% DMSO. The other four plates did show there was a significant difference between the Pd bis-dppz treatment group and the 1% DMSO group.

Investigating Mechanisms of Cell Death

TUNEL Assay

All of the samples treated with the experimental compounds exhibited fluorescent cells when viewed using the fluorescein channel. The amount of fluorescence of both cells treated with the Pd mono-dione and with the Pt mono-dione was equivalent to the amount of fluorescence seen in the positive controls.

Negative controls showed no fluorescence. Figure 17 shows a cell on the positive control slide under normal light, and Figure 18 shows that same cell using the fluorescein channel. The fluorescent nucleus of the cell is clearly visible. It appeared that all of the cells attached to the positive control slide also fluoresced.

Figure 19 shows a cell treated with Pt mono-dione under normal light, and figure 20 shows the same cell using the fluorescein channel. Fluorescence appears to be diffused throughout the cell. All of the cells visible under normal light showed similar fluorescence using the fluorescein channel. The cells appeared rounded up, and the nuclei were not clearly visible. The round shape of the treated cells is consistent with the changes in shape observed in other treated cells and shown in figure 13.

The cells treated with the Pd mono-dione exhibited similar results to the cells treated with Pt-mono dione. Figure 21 shows a cell treated with Pd mono-dione under normal light, and figure 22 shows the same using the fluorescein channel. Again, the entire cell appeared fluorescent, and all of the cells visible under the normal light appeared to be visible and fluorescent using the fluorescein channel. These cells were also round, and the nuclei is not clearly visible with normal light.



Figure 17. HTB-126, Positive control, bright light, magnified ~480x.



Figure 18. HTB-126, Positive control, fluorescence-false color, magnified ~480x

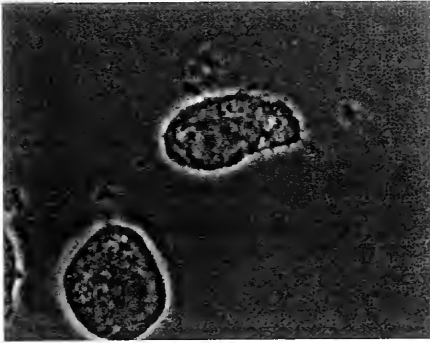


Figure 19. HTB-126 treated with Pt dione, bright light, magnified ~480x.

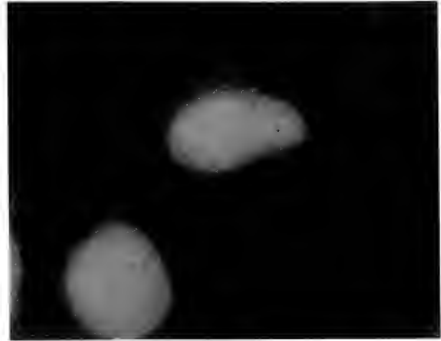


Figure 20. HTB-126 treated Pt dione, fluorescence, false color, magnified ~480x.

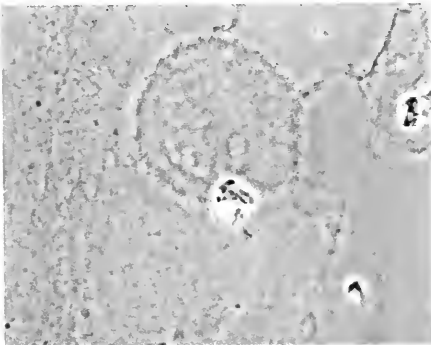


Figure 21. HTB-126 treated with Pd dione, bright light magnified ~480x.



Figure 22. HTB-126 treated with Pd dione, fluorescence – false color, magnified ~480x..

Trypan Blue Assay

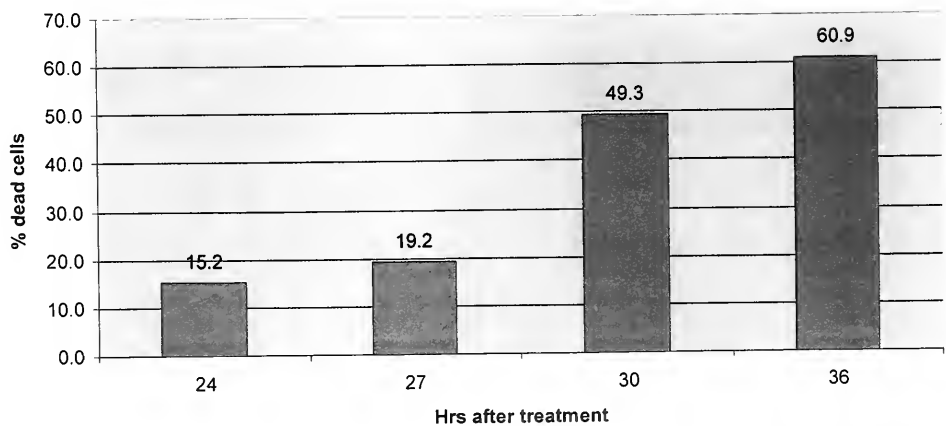
Cells were counted on photos taken during the time course of the experiment. A two inch area was randomly selected to obtain counts of clear cells and blue cells. Three areas were counted for each time interval. Table 7 shows the data from 24 – 36 hours after treatment including the number of unstained cells counted, the number of stained cells counted, the total number of cells counted and the percentage

of cells that were stained in each time interval. Figure 23 shows the averages of the percentages of dead cells in each time period. In all the photos (from 24 to 36 hours of incubation with the Pt mono-dione), all of the cells were round in shape, consistent with all of the other observations of treated cells and seen in figures 13 and 19-22. However, the average percentage of stained cells was fairly low at 24 hours (only 15.2%) and was still only at 60.9 % after 36 hours of incubation with the Pt compound.

Table 7. Trypan Blue Assay Data

time after treatment (hrs)	stained cells	unstained cells	total cells	% stained
36	41	42	83	50.6
36	5	23	28	82.1
36	37	37	74	50
30	26	24	50	48
30	26	19	45	42.2
30	19	26	45	57.8
27	27	12	39	30.8
27	32	5	37	13.5
27	52	8	60	13.3
24	21	5	26	19.2
24	42	5	47	10.6
24	32	6	38	15.8

Figure 23. Cell Death due to Pt mono-dione Over Time



Gel Electrophoresis of HTB-126 DNA

A photograph of the gel is shown in figure 24.

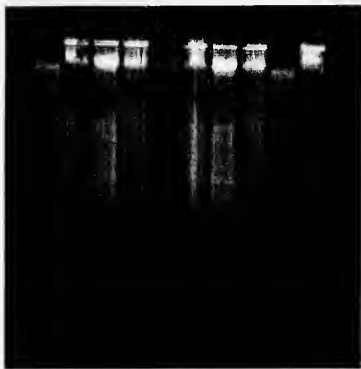


Figure 24. 1.5% agarose gel, photographed with shutter speed 1/8s, and f-stop 4.5

Lane (L-R)	Sample
1	1 μ g 1Kb DNA ladder
2	10 μ g DNA from Untreated HTB-126 cells (Control)
3	10 μ g DNA from HTB-126 cells treated with 10^{-4} M Pd mono-dione
4	10 μ g DNA from HTB-126 cells treated with 10^{-5} M Pd mono-dione
5	0.2 μ g Lambda DNA cut with Hind III
6	15 μ g DNA from Untreated HTB-126 cells (Control)
7	15 μ g DNA from HTB-126 cells treated with 10^{-4} M Pd mono-dione
8	15 μ g DNA from HTB-126 cells treated with 10^{-5} M Pd mono-dione
9	1 μ g 1Kb DNA ladder
10	10 μ g DNA from AHH-1 cells (HMW control)

The notable features of this gel are the faint bands in lanes 2,3,6, and 7. The lanes contain untreated DNA samples and the DNA samples from cells treated with 10^{-4} M Pd mono-dione. Both of these sets of samples seem to have two faint bands occurring in the same places. Some photos of the gel also indicate a faint periodicity of the brightness of the smear in between the clearer bands. Lanes 4 and 8 contain DNA from HTB-126 cells treated with 10^{-5} M Pd mono-dione. These lanes have only a smear with no apparent bands.

The λ DNA was used to determine help determine which bands of the 1 Kb DNA ladder (marker DNA) were evident on the gel. It was decided that the very last band visible in the 1 Kb ladder DNA lane (more apparent in the 9th lane than in the 1st) was the 506/517 bp band. The rest of the bands were then matched to their known sizes according to the product literature (New England BioLabs). The logs of the DNA sizes were graphed (using Microsoft Excel) against the distance migrated and a trendline was added. This graph, shown in figure 25, includes the equation of the trendline. This equation was used to calculate the sizes of the HTB-126 DNA fragments in the band and smears. This data is presented in table 8.

Figure 25. The migration distance versus size of the 1 Kb DNA ladder marker DNA, including the trendline and the equation of the trendline.

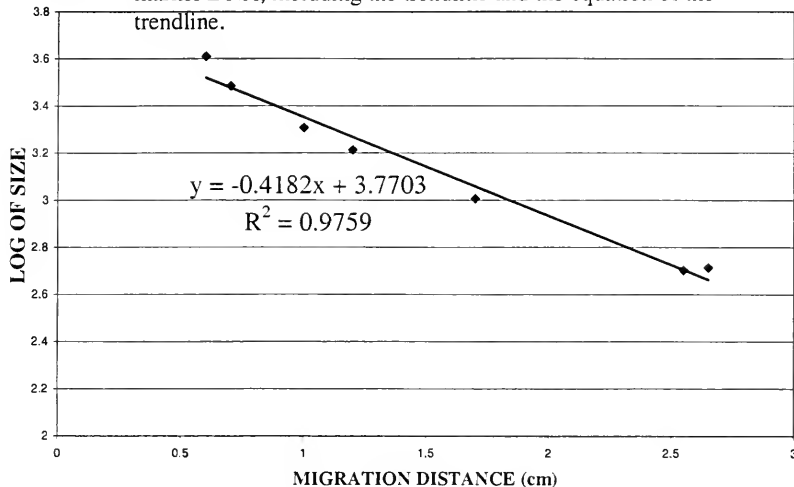


Table 8. Migration distance and sizes of the HTB-126 DNA

Sample	distance	size (bp)
DNA from untreated HTB-126 cells (control)	0.2-0.4	4860-4008
	1.3	1685
	2.15	743
DNA from HTB-126 cells treated with 10^{-4} M Pd mono-dione	0.2-0.4	4860-4008
	1.3	1685
	2.15	743
DNA from HTB-126 cells treated with 10^{-5} M Pd mono-dione	0.2-0.4	4860-4008

Investigating Localization of Cytotoxic Compounds

The attempts to analyze the cell fractions and extracted DNA with atomic absorption spectroscopy were not successful. The instrument was barely able to detect Pd in the 10^{-5} M Pd mono-dione standard and consequently, was unable to detect Pd in any of the experimental samples, or even the lower concentration standards of 10^{-6} M, 10^{-7} M and 10^{-8} M Pd mono-dione. However, some experimental observations made during the sample preparation do give some indication of where the compounds localize.

Before being added to media, the Pd mono-dione solutions are yellow (pale lemon yellow at 10^{-4} M and bright yellow at 10^{-3} M). When cells treated with the 10^{-4} M compounds are detached from the tissue culture flasks, rinsed and centrifuged, the resulting cell pellet is also yellow. Untreated cells yield a white to very pale pink pellet. Furthermore, approximately 20-30% of the isolated nuclei of the cells treated with the higher experimental concentration (10^{-4} M Pd mono-dione for one hour) appear bright yellow when observed with the light microscope.

Discussion:

Comparison of the Cytotoxicities of the Pt mono-dione and Pd mono-dione compounds

Based on the graphs of the average percent survivals of HTB-126 cells treated with the Pt mono-dione or Pd mono-dione solutions (fig. 7) the Pt mono-dione compound appears to be more effective than the Pd mono-dione in killing the HTB-126 cells. For every individual plate, the percent survival of the cells treated with the Pt mono-dione solution is lower than the percent survival of the cells treated with the Pd mono-dione solution. Although this difference varies between plates, the percent survivals are similar between related plates of cells (cells that came from the same flask). The conclusion that the Pt mono-dione is more effective in killing the HTB-126 cells is supported by the statistical analysis. An analysis of the absorbances of this group of plates using a 1 factor ANOVA and the Fischer Protected Least Significant Difference post hoc test shows that the difference between the percent survivals of the Pt mono-dione and Pd mono-dione groups is significant for all but one of the eleven plates tested (table 3, plate B.2). For all eleven plates the statistical analysis showed a significant difference between both the Pd and Pt treatment groups and the control groups treated with 1% DMSO. Since seven of the eleven plates also appeared to have a significant difference between the untreated cells and those treated with 1% DMSO, it is best to compare the Pt and Pd groups solely with the DMSO treated group.

The graphs of the percent survivals of HTB-125 cells (fig.5) also indicate that Pt mono-dione is more toxic than the Pd mono-dione to normal HTB-125 cells. Table 8 (and figure 24) is a summary of the data in figures 5,6 and 7: it averages all the percent survivals for all the plates tested for each cell line.

The averages in this table indicate that both the metal-dione compounds show some selectivity for killing transformed over normal cells. Although the Pt mono-dione appears to be more effective in killing transformed cells than the Pd mono-dione, it is also more effective in killing normal cells. The Pd mono-dione seems to show greater selectivity; it kills a greater percentage of transformed cells in comparison to normal cells. This selectivity may be an important consideration in the development of these compounds into chemotherapeutic treatments, particularly in the efforts to develop a drug that kills cancer cells without killing the patient. Again, for this HTB-125 cell line the Fischer Protected Least Significant Difference statistical analysis (table 1) supports these findings, indicating there is a significant difference between the absorbances of the wells treated with the platinum compound and those treated with the palladium compound, as well as a significant difference between the treated groups and the control groups.

The percent survivals of the CCD-27Sk cells contrast with both the HTB-125 and HTB-126 findings. The graph (fig.6) indicates that the Pt mono-dione compound yielded slightly higher percent survivals, but the statistical analysis (table 3) showed no significant difference between these two treatment groups. This data actually serves to support previous findings (Easterly, 2000; Vogler, 2000) that the compounds affect different cell lines differently. While initial research on these different Pt and Pd complexes indicated that Pd complexes were more toxic than the Pt complexes to most cancer cell lines, Vogler's research suggested that the platinum complexes were slightly more toxic to some cell lines than others (Davies, 1999; Vogler, 2000). The CCD-27Sk cells came from a different person, so is likely the cells have a different biochemistry, which would explain why their reactions to the compounds are different from those of the HTB-125 and HTB-126 cells. The results of this experiment support the conclusions that the identity of the central metal of the complex affects its cytotoxicity and that the effect of each metal varies between cell lines.

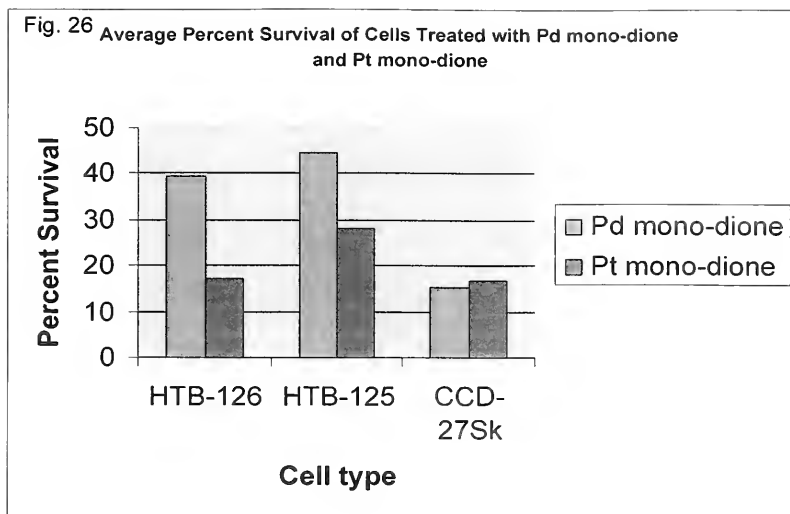
In addition to the quantitative results obtained, the photographs (figures 11-16) of HTB-125 cells treated with the Pd mono-dione and Pt mono-dione solutions suggest some interesting conclusions. Cells

treated with Pt mono-dione (fig. 13) appear completely rounded, indicating they are dead. The cells are also noticeably smaller in volume than normal cells. In contrast the cells treated with Pd mono-dione (fig. 12) appear to have lost their elongated shape and gotten rounder, but not nearly as shrunk as the cells treated with Pd mono-dione. While both figures 12 and 13 show cells clearly in crisis, the cells look very different, suggesting that the two different compounds may have a different mechanism of interaction with the cells. This would explain the quantitative data of the differing cytotoxicities and suggests an explanation for cytotoxicity results varying by cell line.

Although the cells treated with Pd mono-dione look different from the cells treated with Pt mono-dione in these photos, the stages of cell death at the time the photos were taken might not be an endpoint. Other observations during this investigation have indicated that the final appearance of cells treated with the Pd mono-dione is similar to that of the cells in figure 13. These photos were taken of cells that had been exposed to the compounds for the same amount of time, and its possible that this is an indication that the cells treated with Pt mono-dione are not dying differently but rather dying faster than the cells treated with Pd mono-dione. If this is true, then the mechanism of intracellular action might not be different, but the way the compounds are taken up by the cells might be different. If more of the compound is getting into the cell faster, it is likely that that cells would die faster. Because the metal ions most likely affect the transport of the complexes into the cell, it would be reasonable to suggest that a different metal could affect uptake. Little is known about exactly how these compounds interact with any cellular components, such as DNA, and this certainly merits further research.

Table 9. Average Percent Survivals of cells Treated with Pd mono-dppz and Pd bis-dppz

Cell line	Pd mono-dppz	Pd bis-dppz
HTB-126	57.4	87.39
HTB-125	89.67	90.95
CCD-27Sk	78.89	84.67



A comparison of the dipyridylphenazine compounds

The dipyridylphenazine compounds (Pd mono-dppz and Pd bis-dppz) had particularly interesting results. These compounds were created in the hopes that the larger molecules would interact more strongly with the cellular DNA, causing greater disruption and hopefully greater cytotoxicity. However, both Pd mono-dppz and Pd bis-dppz showed significantly less activity than the other compounds tested, although they were not directly compared. In addition, the larger molecule, Pd bis-dppz was almost entirely ineffective in killing tumor cells or normal cells (see table 9). Preliminary observations that both compounds were difficult to dissolve in DMSO and appeared to be suspended rather than dissolved in the solvent suggested an explanation for this seeming lack of activity: perhaps the compounds were not soluble enough to be taken into the cells. Photographs taken of HTB-125 cells treated with Pd mono-dppz (fig 14-16) confirm this hypothesis. Under 352x magnification (fig. 15-16), tiny linear crystals, presumably undissolved compound, can clearly be seen outside of the cells. This insolubility makes it difficult to determine exactly how much compound was actually available to interact with the cells, and

if the concentration of the compound might have varied between trials. That statistical data for these compounds indicated that there were significant differences in the results even between related plates (plates of cells taken from the same flask). This made the results somewhat inconclusive, since both the statistical (tables 4-6) and graphical (fig. 8-10) analysis indicated that the data was inconsistent. Time constraints prevented further trials in this experiment, which might have yielded more conclusive results. In addition, further research would require that these compounds be fully dissolved in a solvent before application to the cell culture.

More research should also be done on the effects of the compounds on the normal cells; though an investigation on the normal cells was attempted during this experiment, time constraints limited the data. While the data in table 9 seems to indicate that the Pd mono-dppz complex showed some selectivity between normal and transformed cells, the “average” for the HTB-125 cells is only from one plate, so it is unknown whether these results are reproducible.

The Use of Statistical Analysis for Analyzing this Data

This is the first time in the research of these Pt and Pd complexes that any form of statistical test has been applied to the absorbance values. The use of the 1 factor and 2 factor ANOVAs to analyze the data did reveal some new insights into the protocol used in this experiment and into how the data should be interpreted. Two-factor ANOVAs including all of the data for each plate in a trial of a cell line indicated that trying to compare plates from different flasks of cells was often like comparing apples to oranges. This is in part because each flask of cells is treated slightly differently. The amount of time the trypsin solution (used to detach the cells from the flask) is in contact with the cells differs and might change the condition of the cells. The age of the cells, how long they have been confluent in the flask, or how many times they have been sub-cultured may all effect how they respond to treatment.

Among cultures of transformed cells it is possible that sub-populations with slightly different mutations exist. These cell lines come from cultures representing a population of mixed cells to start with and have been grown for over 50 passages in artificial conditions with no selection other than those

conditions. It is quite likely that mutant cells that might arise and persist in the populations and that flasks might contain differing subpopulations. Because of potential for such variation, the procedure was modified to focus on testing groups of plates that all came from the same flask of cells. The statistical analysis then focused on one-factor ANOVA's and Fischer LSD tests performed on each individual plate.

The Fischer LSD test also yielded some interesting data about the control groups. Ideally, the untreated cells should have had the same absorbances as the cells treated with 1% DMSO. The Fischer LSD test showed that this was not always the case; some plates had a significant difference between the groups, others did not. However, when a graph of the mean absorbances was constructed including standard error bars, all of the plates that showed a significant difference between the two control groups actually had values that fell within the same range given by that error bars. This raises the question of whether or not the Fischer LSD test might be the best analysis to use on this type of data. However, in all the comparisons of the different complexes, the results matched up well with the graphical data. Nonetheless, further investigation into statistical analysis of this data may prove useful.

Mechanisms of Cell Death

Morphology

Kerr first described apoptosis as "shrinking necrosis" (Kerr & Harmon 1991). Cell shrinkage is probably the most obvious morphological characteristic of a cell undergoing apoptosis. Early photographs taken during this investigation indicate that cells treated with these compounds do indeed shrink as they die. Figure 13 shows HTB-125 cells treated with Pt mono-dione as small, round, and barely recognizable as cells. This contrasts greatly with the size and shape of the untreated HTB-125 cells in Figure 11, a photograph taken at the same magnification. It does appear that the cells are losing volume as they die. While using Trypan blue to assess membrane integrity, it was observed that only cells that had shrunk and become round would take up the dye. This would suggest that loss of integrity of the cellular membrane happened long after other morphological changes indicated that the cells were

dying. Loss of membrane integrity is a very late stage event in apoptosis and may occur only in cells in culture (*in vivo*, apoptotic bodies would be phagocytosed) (Kerr & Harmon 1991). Loss of membrane integrity is not as incidental in necrosis as it is in apoptosis, and is more related to the internal events (ie. swelling) that occur early in necrosis (Barry et al., 1990). The observation of late loss of membrane integrity would suggest the cells weren't dying by necrosis, and were instead following a cell-death timeline consistent with cells undergoing apoptosis

Biochemical Indications of Apoptosis

TUNEL Assay

The results of the TUNEL assay suggest that the DNA of cells treated with both Pt mono-dione and Pd mono-dione has been fragmented, as indicated by the observed fluorescein fluorescence. The breakdown of DNA into nucleosomal fragments is an indication that the treated cells may be undergoing apoptosis. It is significant to note that the fluorescence seen in the treated cells differs from the fluorescence seen in the positive control. The DNA in the positive control was fragmented using DNase, and the resulting fluorescence was localized in the nucleus. The fluorescence in the treated cells was not localized; at the excitation wavelength, the entire cell fluoresced. This would suggest that the DNA fragments of the treated cells are no longer localized to the nucleus. Several sources (Barr and Tomei, 1994; Kerr et al., 1994; Wilson and Potten, 1999) suggest that nuclear disintegration is a hallmark of late-stage apoptosis and occurs at the same time as or just after DNA fragmentation. The TUNEL assay was performed on cells that had been exposed to the experimental compounds for 20-30 hours, which may be an unnecessarily long incubation period. Performing the assay on cells treated for different times would help determine at what point in time the DNA degradation begins and peaks, and would help elucidate the mechanism of cell death observed here.

Gel Electrophoresis of the HTB-126 DNA

The bands and faint periodicity seen in lanes containing DNA from the untreated cells and the cells treated with 10^{-4} M Pd mono-dione is consistent with the appearance of the DNA ladder characteristic of

apoptosis (Gavrieli et. al 1992; Barry et al. 1990). However, the size of the fragments, occurring around 1600 and 700 bp, is a little higher than is usually observed (Gavrieli et. al 1992). Certainly, the banding pattern was unexpected in the lanes containing DNA from untreated cells. It is possible that the untreated cells were undergoing apoptosis prior to DNA extraction. Hypothermia can induce apoptosis, and it is possible that the cells sat too long on the benchtop during the extraction procedure. Another likely explanation is that the 1% DMSO that the control cells were treated with may have induced apoptosis in these cells. This would be consistent with the observations of the MTT assay and the statistical data that showed a significant difference between the survival of cells treated with 1% DMSO and the untreated cells that were used as controls for the cytotoxicity assay.

The major problem with this experiment was that the time frame for DNA fragmentation in these cells was unknown. The DNA used in the electrophoresis was originally intended to be analyzed by atomic absorption, consequently the time of exposure to the cytotoxic compounds was chosen to ensure that the cells had an intact nucleus (the same exposure time was used for cell fractionation as for DNA extraction). In the case of the cells treated with 10^{-5} M Pd mono-dione, the 10 hour exposure time might not have been long enough to observe DNA fragmentation. The absence of banding produced by this sample may simply be a consequence of not exposing the cells to the compounds for long enough. The 10^{-4} M concentration of the Pd mono-dione caused morphological changes and cell death much more quickly than the 10^{-5} M concentration, so the one hour exposure might have been sufficient to produce DNA fragmentation.

Because of the treatment variable involved it was encouraging to see any banding pattern at all in the gel, which supports the TUNEL data. The observation of DNA fragmentation is time dependent, and some cell lines do not produce the DNA ladder although they present the morphological characteristics of apoptosis (Yoshida et al. 1991). Research by Sorenson et al. suggests that detection of fragmentation is time and concentration dependent (1990). Their research suggests that at standard treatment concentrations, the banding produced in gel electrophoresis by DNA fragmentation (from apoptosis

induced by cisplatin) does not peak until 4 days after exposure (Sorenson et al. 1990). Clearly, this is an area in this investigation that would benefit from more research. The timeline of death induced by the novel Pt and Pd compounds has yet to be established, though these studies have made some headway towards that goal. All of these experiments would benefit from comparing different treatment times. The banding produced by the untreated cells also needs to be investigated further. Repeated attempts at gel electrophoresis of DNA from treated cells would probably produce more coherent results.

Location of the Compounds within the Cell

Unfortunately, attempts the attempts to analyze the cell fractions using atomic absorption spectrometry were unsuccessful because the concentrations of the compounds were far too low. Calculations done after the unsuccessful attempt indicate that the 10^{-5} M concentration standard had a palladium concentration of 1.06 ppm. Dr. Ganicott's atomic absorption spectrometer has a sensitivity of 1-15 ppm. If this approach is going to be attempted again, it might be necessary to find a more sensitive instrument. Also, the treatment concentrations and the amounts of cells treated would need to be increased. McKeage et al. had success in detecting DNA platination using flameless atomic absorption spectrometry and DNA extracted from 5×10^7 cells treated with 10^{-5} M concentrations of cisplatin and JM221 (1994). This suggests how much DNA would be needed for future experiments using atomic absorption spectrometry on these cells.

Although the quantitative analysis was unsuccessful, the initial qualitative observations of the color of the treated cells does give some indication of where the compounds localize within the cells. It is clear that a lot of palladium is taken into the cells. It is also very clear that large amounts of the compound are accumulating in the nuclei of many of the cells. Although what the compounds are binding to in the nucleus is unknown, this observation does suggest that further investigation should be done and atomic absorption spectrometry reattempted. It seems very likely that the compound could be binding to DNA within the nucleus.

Conclusions

The cytotoxicity studies performed on these novel cytotoxic compounds suggest that the specific metal ion involved may variably affect cytotoxicity. In addition, these compounds appear to be selectively toxic to transformed cells over normal cells. Observations of the nuclei of the treated cells suggest that the Pd mono-dione compound localizes in the nucleus of the cell and may be binding to DNA. The fluorescent cells produced by the TUNEL assay suggest that cytotoxic compounds are inducing apoptosis in the treated cells. This is supported by gel electrophoresis evidence of internucleosomal DNA fragmentation. Overall, these observations suggest that that novel transition metal based compounds synthesized by Dr. Granger may have potential chemotherapeutic applications as antitumor drugs. Their 4+ oxidation state may allow them to be orally administered, providing a distinct improvement to traditional intravenous chemotherapies. These characteristics suggest that the octahedrally coordinated platinum and palladium compounds warrant further investigation.

Further Research

The novel Pt and Pd compounds investigated here are an exciting development and certainly offer a wide range of possibilities for further research. At the undergraduate level, research can be done to further elucidate the mechanism of action of the compounds, what they bind to within the cell, and what the chronology of the events of cell death is. Beyond this work, it is hoped that the compounds may someday reach animal testing and clinical trials.

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References

- Anderson WK, Quagliato DA, Haugwitz RD, Narayanan VL, Wolpert-DeFilippes MK. Synthesis, physical properties, and antitumor activity of tetraplatin and related tetrachloroplatinum(IV) stereoisomers of 1,2-diaminocyclohexane. *Cancer Treatment Reports* 70 (1986): 997-1002.
- Barr PJ, Tomei LD. Apoptosis and it's role in human disease. *BioTechnology* 12 (1994): 487-493.
- Barry MA, Behnke CA, Eastman A. Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochemical Pharmacology* 40 (1990): 2353-2362.
- Barton JK. Metals and DNA: molecular left-handed complements. *Science*. 233 (1986): 727-734.
- Blajeski AL, Kaufmann SH. "Method for detecting proteolysis during apoptosis in intact cells." Apoptosis: A practical approach. GP Studzinski ed. New York: Oxford, 1999. 215-238.
- Boyd MR. "The NCI in vitro anticancer drug discovery screen." Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval. B Teicher ed. Totowa, NJ: Humana, 1997. 23-42.
- "Cell Proliferation Kit I (MTT)." On-line product information. Roche Molecular Biochemicals, 3 Oct. 1999. 25 Oct. 2000 <<http://206.53.227.20/pack-insert/1465007A.pdf>>
- Chemotherapy for Cancer. <http://www.acs.ohio-state.edu/units/cancer/handbook/chemo.pdf>. [10 Feb. 2002].
- Cooper, Geoffrey M. Elements of Human Cancer. Boston: Jones and Bartlett, 1992.
- Davies, Robin L. "Investigations of Cytotoxicity by Novel Diimine Complexes of Platinum (IV) and Palladium (IV)." [Jeffress Research Grant Proposal]. Sweet Briar College, 1999.
- Debatin KM. Activation of apoptosis pathways by anticancer treatment. *Toxicology Letters*. 112-113 (2000): 41-48.
- Easterly, Evangeline. "The Anticancer Effects of Newly Synthesized Platinum/Palladium Compounds." [Honors Thesis]. Sweet Briar College. Spring, 2000.

- Eastman A. Interstrand cross-links and sequence specificity in the reaction of *cis*-dichloro(ethylenediamine) platinum(II) with DNA. *Biochemistry*. 24(1985): 5027-5032.
- Farmer, Peter B. "Cancer Chemotherapy I: Design and Mechanism of Action of Cytotoxic Drugs." The Molecular Basis of Cancer. Ed. Peter B. Farmer and John M. Walker. New York: Wiley-Interscience, 1985. 261-283.
- Fricker SP, Buckley RG. Comparison of two colorimetric assays as cytotoxicity endpoints for an *in vitro* screen for antitumor agents. *Anticancer Research*. 16(1996): 3755-3760.
- Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *The Journal of Cell Biology*. 119(1992): 493-501.
- Giandomencio CM, Abrams MJ, Murrer BA, Vollano JF, Rheinheimer MI, Weyer SB, Bossard GE, Higgins JD. Carboxylation of kinetically inert platinum(IV) hydroxy complexes. An entrée into orally active platinum(IV) antitumor agents. *Inorganic Chemistry*. 34(1995): 1015 – 1021.
- Goddard PM, Orr RM, Valenti MR, Barnard CFJ, Murrer BA, Kelland LR, Harrap KR. Novel trans platinum complexes: comparative *in vitro* and *in vivo* activity against platinum-sensitive and resistant murine tumours. *Anticancer Research*. 16(1996):33-38.
- Gonzalez VM, Fuertes MA, Alonso C, Perez JM. Is cisplatin-induced cell death always produced by apoptosis? *Molecular Pharmacology*. 59(2001): 657-663.
- Granger RM, Davies RL, Sabat M. "Synthesis and Anticancer Activity of Novel Platinum and Palladium Complexes". <http://rgranger.chem.sbc.edu/>. [15 July 2001].
- Guide to Cell Proliferation and Apoptosis. Roche Applied Science Co. http://www.roche-applied-science.com/prod_inf/manuals/cell_man/p06.pdf. [26 April 2000]
- Guzzie PJ. "Lethality Testing." In Vitro Toxicology. SC Gad ed. New York: Raven, 1994. 57-86.
- Johann TW, Barton JK. Recognition of DNA by octahedral coordination complexes. *Philosophical Transactions: Mathematical, Physical and Engineering Sciences*. 354(1996): 299-324.
- Kerr JFR, Winterford CM, Harmon BV. Apoptosis: it's significance in cancer and cancer therapy.

Cancer 73 (1994): 2013 – 2026.

Kerr JFR, Harmon BV. “Definition and incidence of apoptosis: an historical perspective.” Apoptosis: the Molecular Basis of Cell Death. LD Tomei & FD Cope eds. New York: Cold Spring Harbor, 1991. 5-29.

Kratzke RA, Kramer BS. Evaluation of *in vitro* chemosensitivity using human lung cancer cell lines. *Journal of Cellular Biochemistry Supplement*. 24(1996): 160-164.

Makin G, Hickman JA. Apoptosis and cancer chemotherapy. *Cell Tissue Res*. 301 (2000): 143-152.

McKeage MJ, Abel G, Kelland KR, Harrap KR. Mechanism of action of orally administered platinum complex [ammine bis butyrato cyclohexylamine dichloroplatinum (IV) (JM221)] in intrinsically cisplatin-resistant human ovarian carcinoma *in vitro*. *British Journal of Cancer*. 69(1994):1-7.

Nowell PC. The clonal evolution of tumor cell populations. *Science*. 194 (1976): 23-28.

Rosenburg, B. “Clinical Aspects of Platinum Anticancer Drugs.” Metal ions in biological systems: Metal complexes as anticancer agents. H Sigel Ed. New York: Dekker, 1980.

Sorenson CM, Barry MA, Eastman A. Analysis of events associated with cell cycle arrest at G2 phase and cell death induced by cisplatin. *Journal of the National Cancer Institute*. 82(1990): 749-755.

Studzinski, GP. “Overview of Apoptosis.” Apoptosis: A practical approach. GP Studzinski ed. New York: Oxford, 1999. 1-17.

Vogler, Brienne. “Platinum IV and Palladium IV Compounds as Anticancer Agents: A Selective Cytotoxicity Study.” [Honors Thesis]. Sweet Briar College. Spring 2000.

Wei L, Gupt P, Hernandez R, Farhat F. Determination of ultratrace selenium and arsenic at parts-per-trillion levels in environmental and biological samples by atomic fluorescence spectrometry with flow injection hydride technique. *Microchemical Journal*. 62(1999): 83-98.

Wilson JW, Potten CS. “Morphological recognition of apoptotic cells”. Apoptosis: A practical approach. GP Studzinski ed. New York: Oxford, 1999. 18-39.

Wong E, Giandomencia CM. Current status of platinum-based antitumor drugs. *Chemical Reviews*.

99(1999): 2451-2466.

Yoshida A, Shao RG, Pommier Y. "Assessment of DNA Damage in Apoptosis." Apoptosis: A practical approach. GP Studzinski ed. New York: Oxford, 1999. 41-55.

